Argulus

THE ECOLOGY OF A FISH PEST

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A academic essay in Science

Doctoral Thesis

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according to the decision of the Council of Deans
to be defended in public on Friday, 4th July 2008
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by

Peter David Walker

Born on 2nd January 1979
in Blackpool, England

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For my grandparents, Jenny and Eric Walker,
absent from this world but never from my heart

"*The* Argulus foliaceus *is an exceedingly pretty and graceful little animal; and as it can leave the fish on which it feeds, and swim freely in the water, there are many opportunities for watching its gambols through its native element*"

W. Baird (1850)

Baird, W. (1850). *The Natural History of the British Entomostraca.* Ray Society, London, 364pp.

Table of Contents

Chapter 1

General Introduction

"So, naturalists observe a flea
Hath smaller fleas that on him prey,
And these have smaller fleas to bite 'em.
And so proceed ad infinitum."

J. Swift (referenced in Roberts and Janovy, 1996)

The fact that parasitism has evolved in nearly every known phylum emphasises the success of this mode of existence. The vertebrates are the only phylum where there are few examples of species exhibiting a parasitic mode of existence and yet even here there are examples of parasitic behaviour such as the parasitic males of some angler fish species and lamprey eels, which are widely considered to be parasites.

According to Roberts and Janovy (1996), parasitism is essentially a very specific form of symbiosis. The term symbiosis was originally coined by DeBary in 1876 and was used to describe two species that live together. This covered a range of intimate interactions between organisms, including mutualism, commensalism and parasitism. Mutualism (sometimes also referred to as symbiosis) is used to describe an association between organisms in which both organisms mutually benefit from the association, for example, the symbiosis of the hermit crab *Dardanus fucosus* and the anemone *Calliactis tricolor*, where the hermit crab receives benefit from camouflage and protection from predators and the anemone benefits by having easy access to scraps from the hermit crabs meals and also receives protection from certain predators (López-Victoria *et al*., 2004). Commensalism is the term applied when one member of an association benefits without significantly harming or helping the other member of the association.

In the case of parasitism only one partner benefits from the association, the parasite, and this is by definition at the expense of the other partner, the host. Typically, this gain is in the form of nutrition, but often parasites will benefit in other ways such as shelter from the external environment and protection against predators. Parasites often have deleterious effects on their host's physiology, sometimes even killing the host. However, a ‘good parasite’ should avoid inducing the mortality of its host to minimise the difficulties associated with having to locate a new host (except in some cases where host mortality facilitates parasite transmission). To avoid confusion we will adopt the same definition as Kearn (2004). The term ‘parasite’ throughout this thesis refers only to unicellular or multicellular eukaryotic animals that derive benefit from a symbiotic relationship at the expense of their host.

According to definitions by Roberts and Janovy (1996), a parasite living on the surface of its host is referred to as an ectoparasite but if it lives inside its host it is termed an endoparasite. Some authors also refer to a third group of parasites, mesoparasites, which live in the external openings of an animal's body e.g., the buccal cavity or the cloaca.

Parasites are, in the main, obligate parasites, meaning that they cannot complete their lifecycle without spending at least some part of it in a parasitic relationship with another organism. Some animals can also become parasitic “accidentally” when they enter the body of

another organism via a wound or other opening e.g. the mouth if they are eaten. Parasites can be permanent, temporary or intermittent, depending on whether they spend all, part or repeated short periods in contact with their host.

Intermittent parasites are sometimes termed micropredators. In the case of many ectoparasitic arthropods the term micropredator is perhaps more appropriate than intermittent parasite. Zelmer (1998) provided an evolutionary definition of parasitism which states that the parasite requires the host as both a source of nutrition and a habitat. Ectoparasitic arthropods usually attach themselves to the integument of their hosts in order to feed. However, many of these species can in fact survive quite effectively as independently free-living animals and depend upon a host purely for nutritional reasons. Branchiuran fish lice, the subjects of this thesis, can survive for as long as two weeks without a host animal (see Chapter 6 of this thesis). Due to this lack of dependence upon the host for anything other than nutrition and because many of these intermittent parasites feed on multiple host species, the term micropredator is probably the most accurate. This equating of ectoparasites with predators was also discussed by Ewald (1995).

An advantage of this micropredator lifestyle as opposed to a more permanent parasitic mode of existence could be that the micropredator avoids the consequences of the host's immune response. This can be achieved either by relocating to a site distant from the first feeding site on the same host or, if the immune response is severe enough to be spread systemically throughout the host, by relocating to a new host.

Based on the evidence that endoparasites reach their internal infestation sites by penetrating the host's skin or gills or via ingestion with the host's food, Kearn (2004) suggests that some endoparasites may well be descended from parasites living on the skin or gills of the host animal. He therefore states that “a thorough knowledge of the biology of external parasites of fishes is important if we are to understand how parasites evolve and progress”. It is also plausible that many parasite species are in fact descendents of micropredators that have simply evolved to maintain contact with their prey instead of constantly facing the problems associated with foraging.

Parasites are studied for several reasons but one of the main driving forces behind parasitology studies is the effects they have on humans. This can be either directly in the case of parasites infecting humans or indirectly by infecting livestock in both terrestrial and aquatic farming operations.

With the ever increasing diversification and intensification of aquaculture practices parasitic diseases of fish and shellfish have inherently received an increase in attention due to

the deleterious effects these pathogens can have on farm stocks. Whilst viral and bacterial pathogens have in general received more interest from researchers than other parasitic organisms, the often dramatic effects of salmon lice (*Lepeophtheirus salmonis*) on salmon cage farm stocks have resulted in ectoparasitic lice (particularly copepods) receiving more concentrated research focus. Recent research has also suggested that lice originating from farmed salmon may cause localised extinction of wild pink salmon stocks (Krkošek *et al*., 2007; Rosenberg, 2008).

Much of the research focus has, in the past, centred around the effects parasites have on their hosts and on the development of methods to treat or immunise fish against infection. As such, for many common parasites of fish, there is a general paucity of information regarding the ecology of parasites in their natural environments, particularly when considering the factors which may affect their success as organisms in their own right.

Within European freshwater bodies, *Argulus* spp. are perhaps the parasite species most likely to be encountered by the naturalist, angler or aquarist working in freshwater environments. Despite this there are still huge gaps in our knowledge about these common animals. In 1982, Fryer stated that the alien invader *Argulus japonicus* was already widespread across continental Europe and was likely to be found in the British Isles in the future. His predictions turned out be correct and *A. japonicus* was recorded in Britain for the first time in 1992 (Rushton-Mellor, 1992). Originating in the Far East, this species probably spread with the trade in cyprinids such as carp (*Cyprinus carpio* L.) and goldfish (*Carrassius auratus* (L.)). It is likely that with the onset of global warming this species distribution will continue to expand (Chapter 6 of this thesis).

This thesis aims to expand the current knowledge on branchiuran fish lice with a particular emphasis on the native European species *Argulus foliaceus* (L.) and its invasive relative *A. japonicus* Thiele, by examining key aspects of their life history as shown in Figure 1.

Chapter 2 begins by providing an overview of the literature currently available regarding this group of parasites covering topics from life history traits, morphology, host location and host choice to the effects the parasites have on their hosts in terms of behavioural changes and activation of the stress and immune response.

Chapter 3 details a study which focussed on the occurrence of the different life history stages (i.e. larval, juvenile and adult) of a native European argulid, *A. foliaceus*, on different host species found in a commercial coarse fishery. This chapter aimed to answer two main questions:

1) Are some fish species more likely to be infested with *A. foliaceus*?
2) Are larval, juvenile and adult lice distributed similarly within the host community?

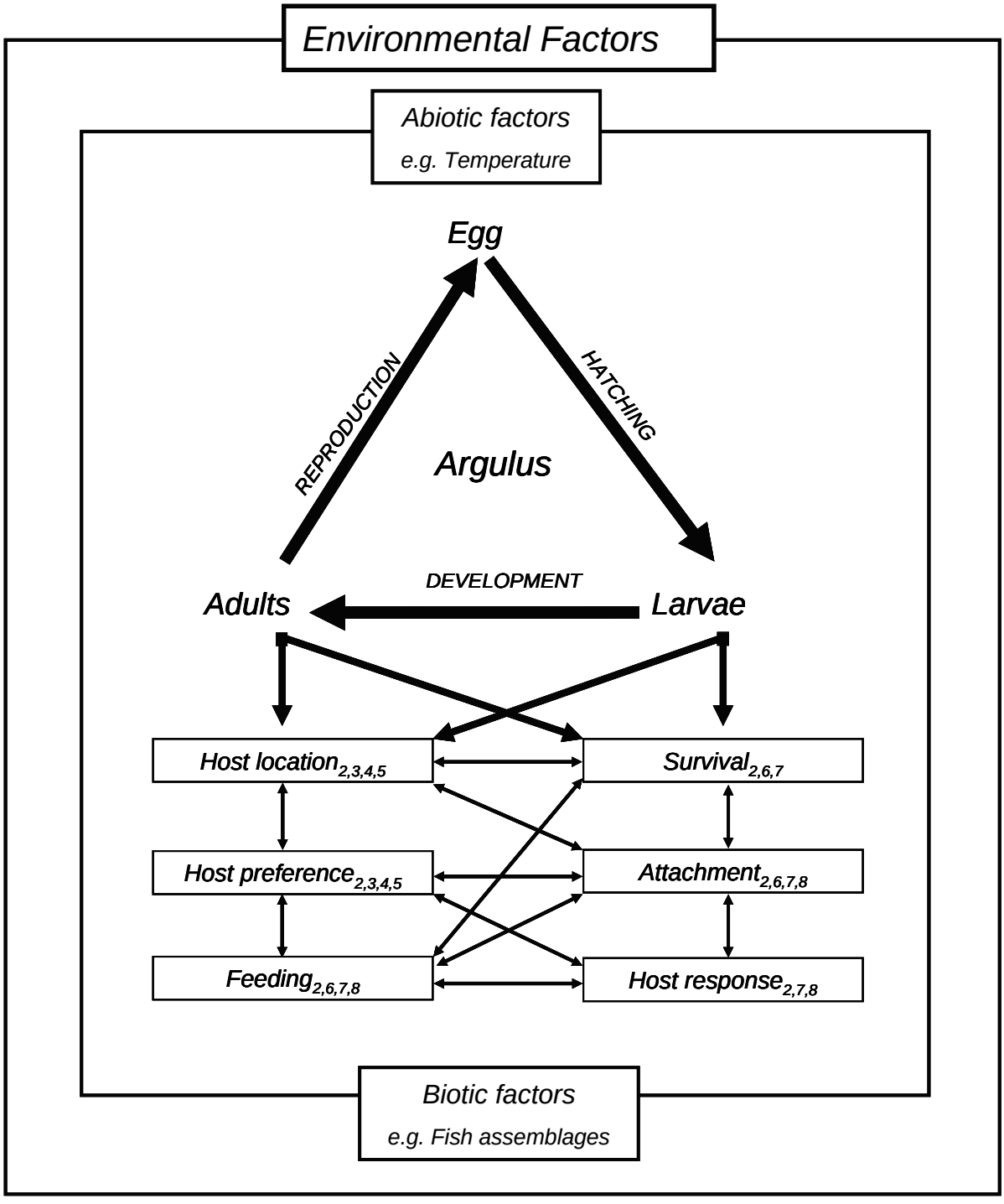

Figure 1. Schematic representation of the way the environment (both abiotic and biotic) influences the distribution of a generalised argulid via effects on its behaviour, ecology and physiology. Key aspects of the life history between larval hatching and adult maturation/reproduction and how these aspects are interrelated are shown. Numbers given in text boxes correspond with chapters in this thesis, where each of these aspects is addressed to some extent.

Chapters 4 and 5 further explore the infection dynamics of *A. foliaceus* amongst a wild fish community by looking at the effect of host size on parasite infection intensity and prevalence. The third question of this thesis, which is addressed in these two chapters, is:

3) Are larger fish more heavily infested with *A. foliaceus* than smaller fish?

The mechanisms and circumstances that result in epizootics of many parasite species has been a topic of great interest to parasitologists, but for the vast majority of parasite groups our understanding is still limited. With the continuing onset of global warming it is certain that we will observe changes in the parasite fauna, especially in aquatic environments. As such it is vital that we increase our understanding of the way temperature affects parasitic organisms. Chapter 6 examines the effect of temperature on the off-host survival of three life history stages of the exotic *A. japonicus* and native European *A. foliaceus*. This chapter also examines the effect of starvation and temperature on the viability of *A. japonicus*. Here we attempt to answer the following questions:

4) Does temperature affect the off-host survival time of *A. foliaceus* and *A. japonicus*?
5) What are the maximum off-host survival times of these two species?
6) Does temperature affect the attachment success of *A. japonicus*?
7) What are the effects of starvation on the attachment success of *A. japonicus*?

Once argulid parasites have successfully located a host and have attached to it they can begin to feed. Chapter 7 reviews the mechanisms by which these animals feed using *A. japonicus* as a model. The main question we aim to answer in this chapter is:

8) What is *A. japonicus*'s diet?

Advances in the field of fish immunology have been significant in recent years. Our understanding of the way in which fish respond to infection from pathogenic organisms is increasing all the time due mainly to the advancement of the techniques used to study immune responses. In chapter 8 we use standard histological techniques to illustrate the damage caused by the feeding activities of *A. japonicus*. In addition we use PCR techniques to demonstrate the immune response of common carp to infection by *A. japonicus* at the molecular level. The question addressed in this chapter is:

9) Does *A. japonicus* elicit an immune response in common carp?

In Chapter 9 the main findings in this thesis research are discussed in relation to the questions introduced in this introductory chapter.

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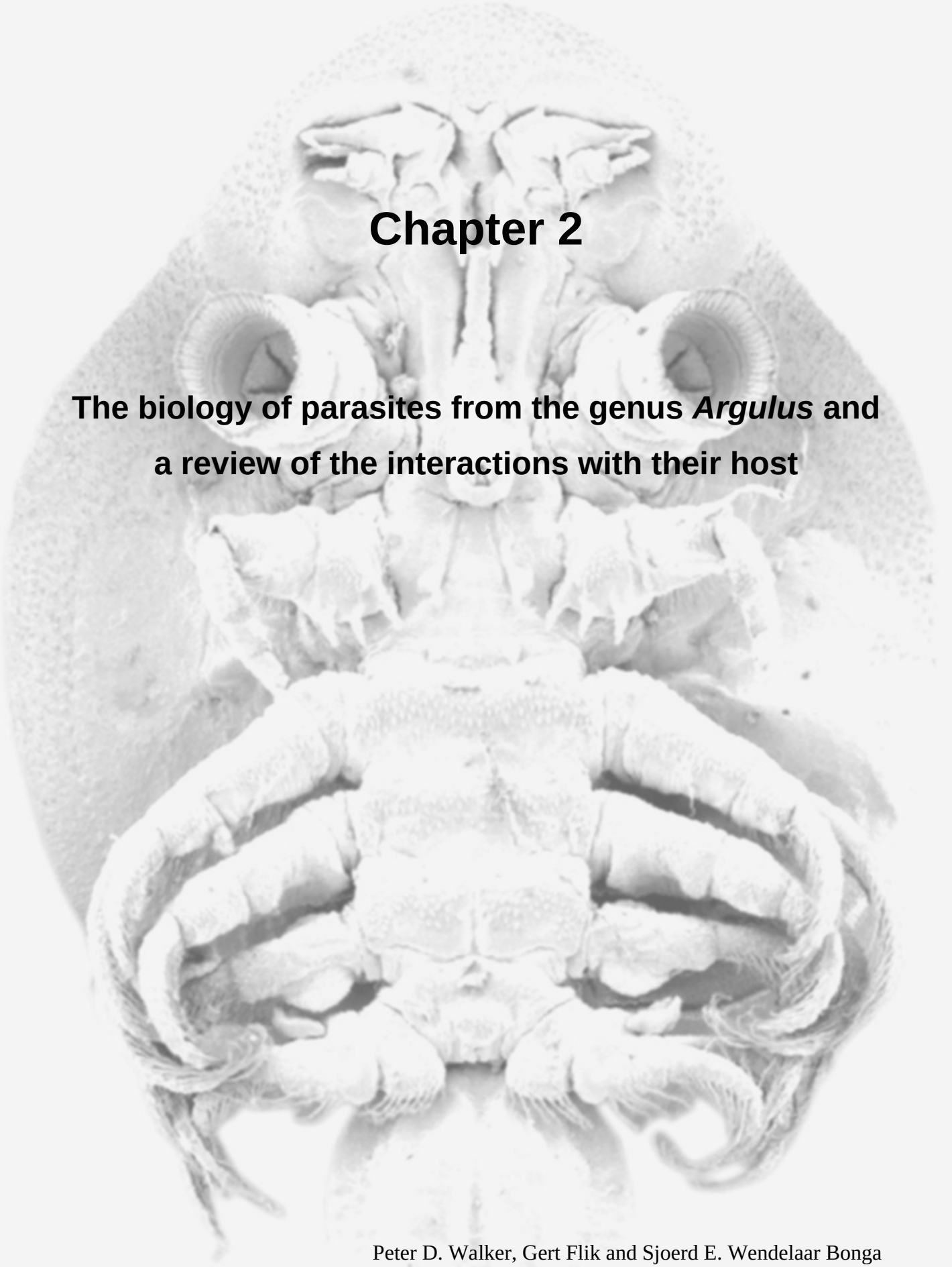

Chapter 2

The biology of parasites from the genus *Argulus* and a review of the interactions with their host

Peter D. Walker, Gert Flik and Sjoerd E. Wendelaar Bonga

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1. Introduction

During the 20th century crustacean ectoparasites of fish did not receive the same research focus that other piscine pathogens experienced. This has resulted in a delay in our understanding of these economically important organisms. However, it is now recognized by fin fish producers and researchers alike that parasitic lice can indeed play a significant role in the economic success of aquaculture organizations. Around the globe there are continuous reports on the deleterious effects of these pathogens on fish farm stock (e.g. Bauer, 1959; Costello, 1993; Menezes *et al*., 1990; Revie *et al*., 2002; Saksida *et al*., 2007) and on wild fish populations (e.g. Poulin and Fitzgerald, 1987, 1988; Johnson *et al*., 1996; Whelan and Poole, 1996; Todd *et al*., 2000; Krkošek *et al*., 2006). A recent report even showed that lice may be causing localised extinctions of some fish populations (Krkošek *et al*., 2007). Even parasitic infections in recreational fisheries are under current investigation to evaluate the effects of such infections on fishery economics (Taylor *et al*., 2006). These reports have led to an increase in the number of researchers concentrating on the study of ectoparasitic crustaceans found on fish but there is still a considerable lack of knowledge in some areas. This chapter aims to give a review on the current ecological knowledge available for the Branchiuran genus *Argulus* including a focus upon the general biology and parasite-host interactions.

All argulids are described as obligate ectoparasites of fish but they are also frequently encountered swimming freely in the water column as they seek out new hosts, mates or when females detach from their hosts to deposit eggs (Bower-Shore 1940; Mikheev *et al*., 1998; Bandilla *et al*., 2007a). Morphologically they bear a close resemblance to several parasitic copepod species and this similarity has led to some conflict over their classification during the 20th century (Martin, 1932; Kearn, 2004). To date the majority of researchers are in agreement that similarities exist as a result of convergent evolution rather than the sharing of a common ancestor (Kearn, 2004). Table 1 details the taxonomic classification of the genus *Argulus* according to descriptions by Bowman and Abele in 1982.

When factors such as parasite distribution and relative lack of host specificity are considered, the genus *Argulus* can be regarded as one of the most widespread and economically important groups of crustacean ectoparasites affecting freshwater fish around the globe (Bower-Shore, 1940; Menezes *et al*., 1990; Shafir and Oldewage, 1992; Taylor *et al*., 2006). Whilst morbidity is not always linked to infections of *Argulus* spp., the direct and indirect results of louse infections can still be significantly costly to aquaculture and sport fishing operations (Menezes *et al*., 1990; Northcott *et al*., 1997; Taylor *et al*., 2006).

Table 1. Taxonomic classification of the genus *Argulus* (After Bowman and Abele, 1982)

Taxonomic Level	Taxa
Phylum	Arthropoda
Subphylum	Crustacea Pennant, 1777
Class	Maxillopoda Dahl, 1956
Subclass	Branchiura Thorell, 1864
Order	Arguloida Rafinesque, 1815
Family	Argulidae Leach, 1819
Genus	*Argulus* O. F. Müller, 1785

In addition to the deleterious impacts resulting from parasitic feeding and attachment, secondary infections from bacteria and fungi (Stammer, 1959; Shimura *et al*., 1983; Singhal *et al*., 1990) are very common and argulids have also been shown to act as vectors for other pathogens including nematodes (Moravec, 1994; Molnár and Székely, 1998) and viruses (Dombrowski, 1952; Ahne, 1985; Cusack and Cone, 1986).

There are several reviews available for argulids concentrating on factors such as distribution (Gurney, 1948; Rushton-Mellor, 1992; Poly, 1997, 1998), development (Rushton-Mellor and Boxshall, 1994) and morphology (Martin, 1932; Benz and Otting, 1996). This chapter aims to provide an overview of the currently published knowledge regarding, in particular, those species of *Argulus* found in European freshwaters.

2. Life cycle, growth and development

The life cycle of argulids has been described by several authors (e.g. Bower-Shore, 1940; Kollatsch, 1959; Mclaughlin, 1980; Kearn, 2004) and evidence shows that the cycle is very similar for all members of the *Argulus* genus. The only differences appear to be in the number of developmental stages between hatching and maturity. Figure 1 illustrates the key stages involved in the life cycle of a generalised argulid.

Eggs are deposited on a suitable surface (e.g. plant stems, stones or glass sides of aquaria) in clumps or more typically parallel rows (Fig. 1), a process reported to be unusual for aquatic ectoparasites (Williams, 1997). In parasitic copepods (e.g. *Caligus elongatus, Lepeophtheirus salmonis, Tracheliastes maculatus*) the females generally possess egg sacs in which eggs are maintained until hatching (Walker *et al*., 2006). The number of eggs deposited by any female argulid varies considerably between individuals (from less than 10 up to several hundred) and to date there is no conclusive evidence regarding the factors affecting

clutch size. Even between different species from this genus there are no obvious differences between clutch sizes. We speculate that gravidity is dependent upon such factors as meal quality (i.e. health status of host fish), parasite size (or age) and parasite species. Development time of egg stages is heavily dependent upon the temperature of the surrounding water with development being more rapid at higher temperatures. *Argulus foliaceus* eggs for example hatch after just 8 days at 26^{o}C (Fryer, 1982) or after several months for eggs deposited at temperatures below 10^{o}C (e.g. Lester and Roubal, 1995; Mikheev *et al*., 2001). This could be a strategy that evolved to enable parasites in temperate regions to survive harsh winter conditions as an egg stage and to take maximum advantage of favourable summer conditions to increase and/or maintain population sizes (Fenton *et al*., 2006). Certainly overwintering egg stages have been reported for several species of this genus (e.g. Bower-Shore 1940; Mikheev *et al*., 2001).

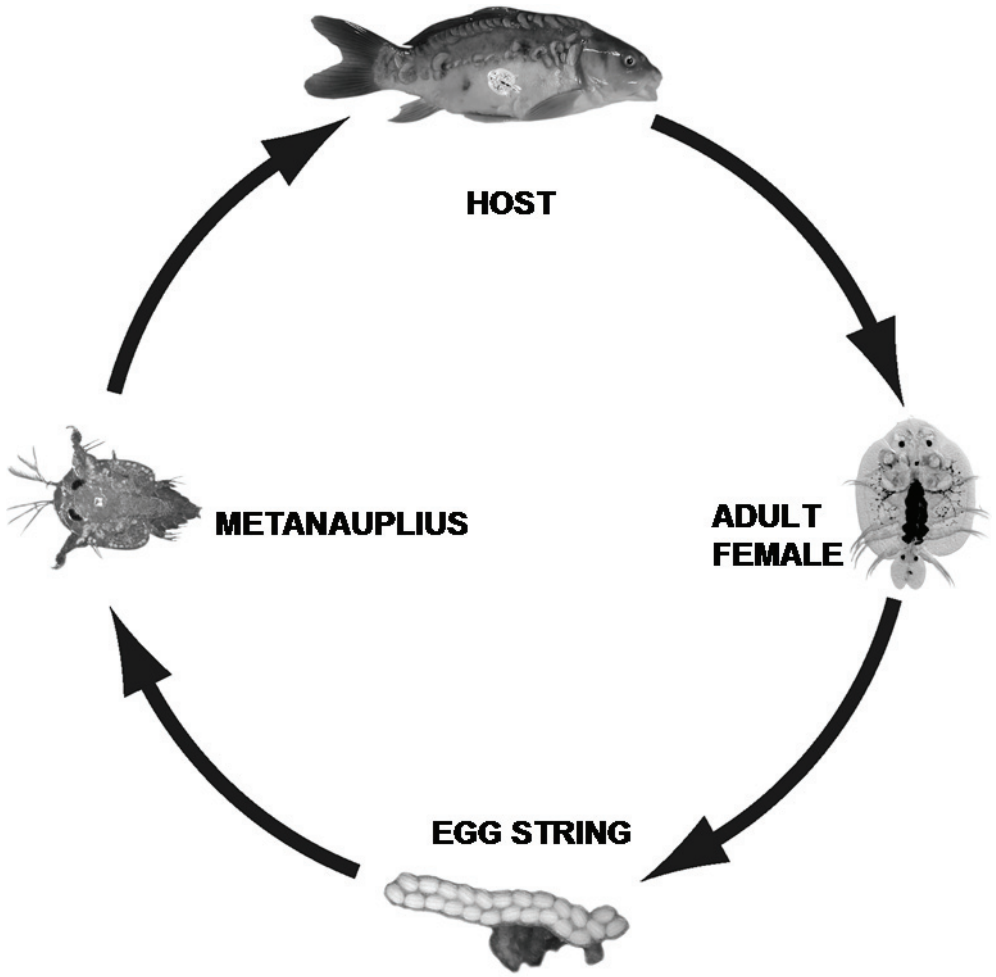

Figure 1. Generalised life cycle for the genus *Argulus*. Copulation occurs on the host after which the female detaches to deposit eggs in rows or clumps on a suitable substrate (e.g. a rock, plant stem or even glass sides of aquaria). The tiny larvae are immediately parasitic and upon locating a suitable host they undertake several moults until the adult stage is achieved. Adult lice may continue to moult throughout their life.

Upon hatching, the late nauplius larva (Fig. 1) resembles the larger adult form. Although the general body forms of the different life history stages are similar, there are distinctive morphological features that enable differentiation of the various developmental stages (Rushton-Mellor and Boxshall, 1994). The copepodid-like metanauplius larvae are immediately parasitic (Lester and Roubal, 1995) and perish within a few days of hatching if a suitable host is not located (Kollatsch, 1959; Chapter 6). After just a few days of feeding these hatchlings moult into a second stage which possesses most of the features of an adult and can therefore be referred to as juveniles (Rushton-Mellor and Boxshall, 1994). A key morphological difference between juvenile and adult lice is a lack of the prominent maxillary suckers, which only begin to appear in later developmental stages. A succession of moults takes place approximately every 5 days depending on the individual species and the ambient temperature (Fryer, 1982). The total number of developmental stages involved is species-dependent but examples include 7 stages for *A. japonicus* (Tokioka, 1936), 9 stages for *A. coregoni* (Shimura, 1980) and 10 stages for *A. foliaceus* (Rushton-Mellor and Boxshall, 1994). After approximately 4 to 6 weeks a mature adult louse is recognisable. This development time is generalised here for ease of explanation but the reader should be aware that this period can be significantly shorter or longer depending upon temperatures and individual *Argulus* species examined (see Hindle, 1949; Shimura, 1980; Fryer, 1982).

Although all argulid species are dioecious, i.e. have separate sexes (Benz and Otting, 1996; Pasternak *et al*., 2004), the sexes do not differ enormously in terms of their morphology. Separate sexes are distinguishable by examination of the abdominal lobes located at the posterior end of the parasite's body. Females possess small spermathecae whereas males possess large testes, which are clearly visible in live specimens due to the transparent properties of these animals' exoskeletons. In addition, white eggs can often be seen within the pigmented ovaries located along the midline in adult, female lice. Copulation typically occurs on the host although our observations have revealed that lice will also copulate whilst detached from their host fish i.e. whilst swimming freely in the water column. Copulation involves the transfer of sperm from the male directly to the female. Sperm cells are then stored in the female's spermathecae until she fertilises her eggs during the deposition process (Kollatsch, 1959). Eggs are typically protected by a mucous-like coating that presumably protects them from some smaller predators or opportunistic bacteria and fungi or possibly plays a role in maintaining the hydro-mineral balance of the fertilised eggs.

3. Morphology

Argulid morphology has been the topic of attention for several authors (e.g. Martin, 1932; Bauer, 1959; Kabata, 1985; Wadeh *et al*., 2007) and in light of this only a superficial summary of those structures considered important to the parasite's life style and ecology are described here along with some discussion of their possible function. Similarly, a detailed description of the morphology of larval and intermediate juvenile stages is also omitted here. Figure 2 shows the key morphological features of a typical argulid. Much of an argulid's morphological design can be linked to its ectoparasitic life style. The dorso-ventrally flattened body covered by a large, rounded carapace presents a streamlined surface offering little resistance to water currents that may otherwise dislodge a parasite when a host fish moves through the water column. This general form can also be witnessed in other ectoparasitic organisms of host animals such as fish and birds that need to maintain a streamlined form, e.g. *Lepeophtheirus salmonis* (salmon louse), *Caligus elongatus* (sea louse) and *Crataerina melbae* (swift louse/flat fly). Such a large number of parasites demonstrate this ectoparasitic mode of existence and exhibit this flattened, streamlined form, that we must consider it to be an evolutionary successful trait.

The cuticle of all argulids, like most crustaceans, is chitinous and forms a rigid exoskeleton that provides the support needed for the animal to maintain its form in a similar way to the internal skeleton of vertebrates. The body can be divided into three distinct regions; i) cephalothorax, ii) thorax and iii) abdomen (Fig. 2). When live specimens are observed one can often see numerous pigment cells (chromatophores) which are typically associated with the gut and ovaries. Respiratory areas can be viewed as those areas of the carapace lacking in the small spines and scales that adorn the ventral surface of the carapace. These areas possess a much thinner cuticle and are located adjacent to a large blood sinus which facilitates the diffusion of oxygen into the blood stream of the animal. The shape and position of these areas is also useful for taxonomic purposes (Benz and Otting, 1996).

3.1 Attachment

Streamlined bodies, however, do not provide all the necessary tools to keep an ectoparasite attached to its host and argulids typically show an array of structures that assist in keeping the parasite connected to its food source (Fig. 2). Not surprisingly the attachment structures are all located on the ventral surface of the animal which is the surface that is in contact with the animal's host organism. The most conspicuous of these structures are the large maxillary suckers. These suckers are actually modified first maxillae (commonly referred to as

'maxillules'). Their chitinous support structures (formed by rods composed of sclerites stacked on top of each other (Benz and Otting, 1996) and associated musculature provide a powerful suctorial action that keeps these animals 'stuck' to their hosts. These suction cups are positioned upon a moveable stalk allowing the parasite to move the suckers independently across the host's surface, and this means that the louse can travel over the body of its host with relative ease and surprising speed. In addition to these highly specialised structures argulids also possess modified first antennae that appear as hooks. Numerous small setae, spines and bristles are also believed to play a role in attachment and may also have a defence purpose. These various spines and scales can be observed on the majority of the ventral surface (Fig. 2).

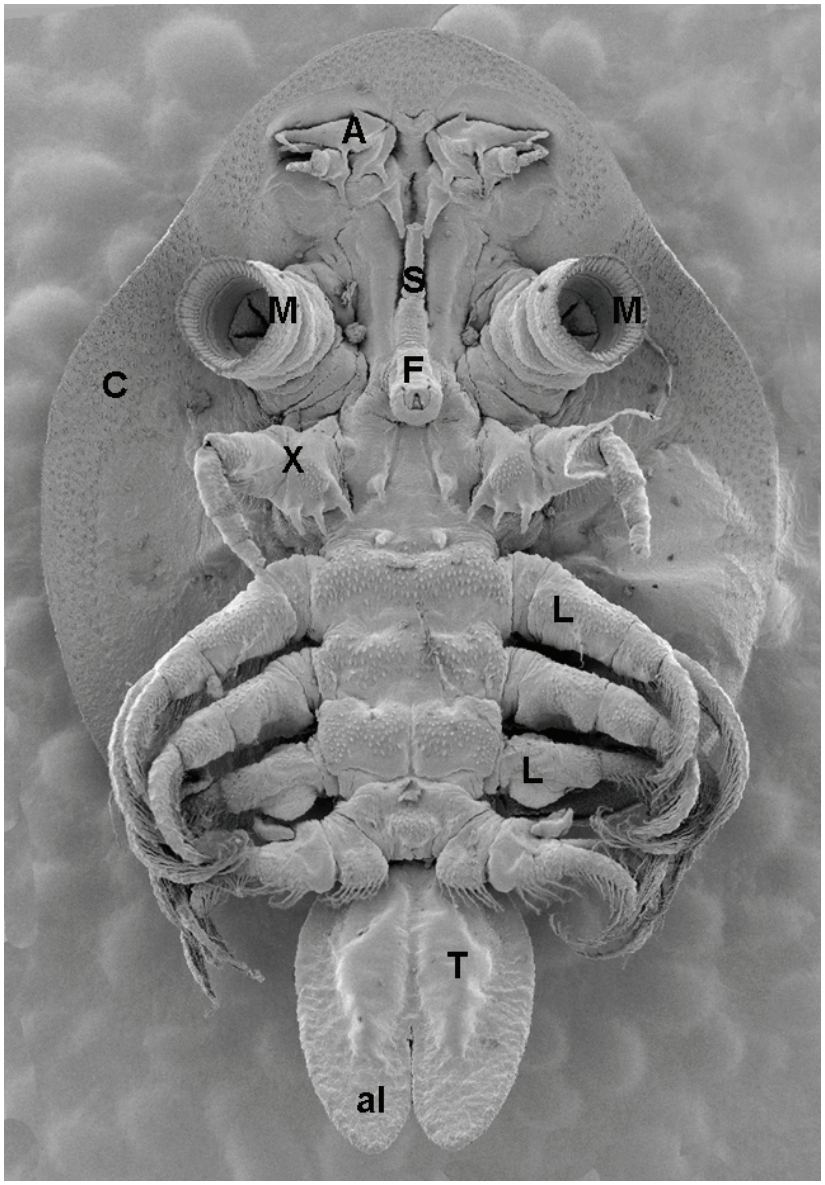

Figure 2 Scanning electron micrograph of the ventral surface of an adult male *Argulus japonicus* (actual size = 6mm). The key morphological features are labelled here and discussed in section 7.3. Abdominal lobe, al; Antennae, A; Carapace, C; Feeding proboscis, F; Maxillary suckers, M; Second maxilla, X; Sheath containing pre-oral stylet, S; Testes, T; Thoracopods (legs), L. Note also numerous small spines and hook-like projections probably used to assist attachment.

3.2 Locomotion

Section 2 discussed the life cycle of these parasites and drew attention to the fact that these animals can and frequently do leave their hosts for a variety of reasons (e.g. accidental dislodging, mate location, egg deposition and new host location). During these 'off-host' periods argulids must propel themselves through the water column with a great deal of efficiency, especially if they are aiming to successfully locate a new host. Indeed, argulids are quite proficient swimmers (Bandilla *et al*., 2007a, b). Propulsion is provided in the main by the four pairs of thoracopods located on the posterior portion of the animal's ventral surface. These appendages exhibit the primitive crustacean form (Martin, 1932) in that they are cirriform and biramous with two segmented sympods and rami, each with lateral rows of pinnate setae (Benz and Otting, 1996). The long setae on these swimming appendages form a paddle-like surface as they beat backwards, propelling the animal forwards. These limbs are also frequently moved backwards and forwards whilst the louse is attached to its host, presumably to provide a continuous flow of fresh water across the respiratory areas. In addition argulids can also 'catapult' themselves very quickly over a short distance using a method similar to that employed by lobsters and shrimps. The lice rapidly flick their abdominal lobes ventrally and towards the anterior of their bodies at the same time as flexing the whole carapace. This action is typically used in predator avoidance although it is possible that lice will also use the same technique to 'jump' onto a passing fish.

3.3 Feeding

The mechanisms employed by argulids to access a meal from their hosts has been described by many authors in the past and yet still remains a topic of debate. This topic will be addressed in Chapter 7 in more detail. In the simplest description the feeding apparatus of an argulid is composed of a retractable pre-oral stylet (also referred to as a sting or stiletto) contained within a sheath and a feeding proboscis or mouth tube (Fig. 2).

The pre-oral stylet is located on the midline of the lice just anterior to the feeding proboscis and in specimens prepared for scanning electron microscopy the stylet may be completely or partially extended or may even be retracted completely inside its associated sheath. According to Martin (1932) the relative size of this stylet varies considerably between species and although often considered as part of the argulid's mouthparts it bears no direct relation to the other mouthparts associated with the proboscis. The proboscis itself also lies along the midline of the animal, just posterior to the aforementioned stylet. When an argulid

is not feeding this proboscis rests in a medial groove but during feeding activities it is extended away from the parasites body so that it meets the host's integument at right angles.

3.4 Sensory organs

The structures/organs utilized by an organism to sense its environment are typically diverse and can take the form of light-sensitive organs (including eyes), mechanoreceptors that detect pressure waves (e.g. the lateral line in fishes) and chemosensory/olfactory organs (including antennae and nostrils).

Argulids certainly possess well-developed compound eyes and several authors (e.g. Madsen, 1964 and Mikheev *et al*., 1998), believe that these function as the main organs utilised by argulids to locate their hosts. A median, naupliar eye is also present but this relatively primitive structure can probably detect nothing more than simple changes in light intensity, which would provide information for the parasite regarding its orientation. In addition, antennae, setae and sensillae can be observed on various parts of the animal's surface and these structures may have a sensory function. Several authors have speculated on the role of mechanosensation and chemosensation in locating hosts and possibly mates (e.g. Galarowicz and Cochran; 1991) and recently Bandilla *et al*. (2007b) demonstrated a positive directional response to chemical cues (fish and mate odours).

4. Distribution and seasonality

One of the factors that make *Argulus* spp. so successful as a parasite and so threatening as a pathogen is its apparent lack of specificity (See Kennedy, 1974; Holland and Kennedy, 1997; Chapter 3) . Argulids are often described as being able to infect any freshwater fish and several species have even been recorded from amphibians, especially tadpoles (Wolfe *et al*., 2001; Poly, 2003). This lack of specificity appears to be one of the key factors that have resulted in the cosmopolitan distribution of many *Argulus* species. In addition some species of *Argulus* can tolerate temperature ranges from as low as 3^{o}C up to 28^{o}C (Chapter 6 and references therein). However, the disease argulosis (the causative agent being *Argulus* spp) is often referred to as being a seasonal disease, particularly in temperate regions. Certainly *A. foliaceus, A. japonicus* and *A. Coregoni* reach their maximum abundance in Europe during the late summer/early autumn months. Relatively few lice are found during the winter and for many years researchers believed that lice populations only survived a winter due to overwintering egg stages deposited in the autumn. Over the last few decades, however, there have been many reports of adult argulids being found on fish sampled during winter months

(Bower-Shore, 1940; Kimura, 1970; Shafir and Van As, 1986). This could be an unnatural phenomenon facilitated by global warming.

5. Parasite-host interactions

In general, parasites are significantly smaller than their host organisms (think of a single flea on a dog). Despite their relatively small size many parasites have the potential to exert strong effects on their host's biology, the consequences of which are often detrimental to the individual host organism. Many parasites are even capable of causing mortality of their hosts although this does not make evolutionary sense because many of them are in fact obligate parasites meaning they depend on their host organisms for nutrition and survival. Still, this 'harm-causing' ability is one of the criteria proposed by Begon *et al*. (1990) as being a defining characteristic of a parasite.

The relationships between host animals and the organisms that parasitize them are very intimate and have typically been subject to generations of co-evolution, which has resulted in the characteristics of infection that we observe. In this section we will discuss several key aspects of the host-parasite relationship between argulids and their fish hosts. We will begin with some of the more ecological aspects of this relationship, e.g. host choice, and then expand to introduce topics such as the immune response to argulids and the stress response of fish to ectoparasites. Due to the embryonic research status of this topic we will also give examples from other host-parasite relationships to illustrate key topics where appropriate.

Whilst argulids are typically described as obligate ectoparasites of fish this description carries some flaws. Argulids are indeed ectoparasitic organisms because they attach to external surfaces of their hosts and feed from this attachment site. It is also fair to state that they are obligate parasites because they must locate a host to feed. However, they are not necessarily obligate parasites of fish. Several species of the genus *Argulus* have been documented from amphibian hosts and we have witnessed *Argulus foliaceus* successfully attaching to *Xenopus laevis* toads under laboratory conditions (*unpublished data*). It is important to note here that argulids can and frequently do detach from their hosts for periods of time making them temporary or intermittent parasites compared to permanent parasites that spend their entire adult lives within or on their hosts (Roberts and Janovy, 1996). For this reason argulids can even be assigned the term 'micropredators', which is a term also used to describe some other temporary ectoparasites such as mosquito's and ticks (Chapters 1 and 2).

5.1 Host choice and specificity

Many species of lice from the genus *Argulus* are described as being non-host-specific. Poulin (1998) defines parasite specificity as 'the extent to which a parasite taxon is restricted in the number of host species used at any given stage in the life cycle' and we will adopt this definition here. In Western Europe there are three known freshwater species from this genus: *A. foliaceus*, *A. coregoni*, and the introduced *A. japonicus*. When examining lists of fish species that these louse species have been found on, then the genus quickly appears to show very low specificity. However, studies have shown that under certain conditions these parasites may show some preference for certain fish over others (Mikheev *et al*., 1998; Kearn *t al*., 2004 *and referenes therein*). For the purpose of this discussion we will concentrate on the common European species because host preference studies are most common for these lice species and they are also some of the more common species known from around the globe.

The *A. foliaceus* population of a small mixed coarse fishery in South-West England underwent an intensive 5 month study to examine population dynamics and infection characteristics (Chapters 3, 4 and 5). A total of 8 fish species were recorded and all of them yielded individual fish harbouring lice at some point during the study. However, the prevalence (proportion of infected fish) and infection intensity (number of lice per fish) differed markedly between individual fish and species, and the infection characteristics varied over the sampling period. During the warm summer months the infection levels (both prevalence and intensity) increased significantly on adult carp (*Cyprinus carpio*). These fish spend a large portion of their time basking in the warmer shallow regions of the lake and as a result may prove to be easier hosts for the lice to locate. Adult bream (*Abramis brama*) showed similar infection characteristics.

A comparative study of argulosis from two sites in South West England revealed differences in preferred host species for *A. foliaceus* (Walker, 2002). The two sites differed somewhat in their fish community structure and species present with one being a densely populated commercial coarse fishery and the other a sparsely populated natural lake with a much greater volume and depth. The results of this study suggested that host choice by argulids cannot be characterised definitively. Host choice instead depends on the combination of several factors such as host species present, population densities and prevailing environmental conditions both in terms of the physical and chemical parameters (e.g. depth, temperature, dissolved oxygen).

Other biological factors may also play a significant role in host selection by argulids. Poulin and Fitzgerald (1988) noted that during daylight hours *A. canadensis* would inhabit the lower half of the water column. Certain fish species (e.g. rudd, *Scardinius erythropthalmus*) spend much of their time in the upper few centimetres of the water column feeding on small zooplankton and other invertebrates that may drop onto the water surface. This would reduce the likelihood of these fish encountering lice at least during daylight hours. In contrast, eels spend much of their time hidden amongst submerged objects or half buried in the bottom silt and again this would reduce their chances of encountering lice. In fact this behaviour may account for the fact that very few eels have ever been found harbouring lice (Evans and Mathews, 2000).

Mikheev *et al*. (1998) noted that light intensity significantly affected the host choice of *A. foliaceus*. In darkened conditions perch (*Perca fluviatilis*) were favoured over roach (*Rutilus rutilus*) and when light intensity was increased roach became increasingly more preferable to the lice. Reduced activity and vertical positioning of the fish probably accounts for some of this variation in host choice, but, the host location strategies employed by the lice suggests something more interesting. During light periods the free-swimming lice spent most of their time hovering almost stationary in the water column whilst during dark periods they appeared to undertake a much more active searching strategy (Mikheev *et al*., 1998). The 'sit and wait' strategy probably serves to conserve energy stores whilst host fish are more active during daylight hours. The active searching strategy would then be more successful during darker periods when many fish lie dormant. This also suggests that argulids can either see extremely well in dark conditions or that they have some kind of chemosensory capability (Bandilla *et al*., 2007). If these lice do indeed have chemosensory capabilities then it is plausible that certain fish species will have a more appealing set of chemical discharges that argulids could home in on. In the case of the experiments conducted by Mikheev *et al*. (1998), perch would have emitted the more appealing 'smell'. Galarowicz and Cochran (1991) showed that *A. japonicus* would indeed respond to host chemical cues, and Y-maze style investigations undertaken by Bandilla *et al*. (2007) substantiate this theory.

5.2 The effects of argulids on fish

The effects of *Argulus* parasites on their fish hosts are quite diverse and range from physical damage caused by attachment and feeding activities of the lice to behavioural changes associated with stress. The following sections will highlight the current knowledge on the effects of the parasites on fish with a particular emphasis on stress and behaviour. Other

topics such as physical damage and immune responses related to infection will also be introduced.

5.2i Effects on host behaviour

Many parasites are known to affect their host's behaviour during the course of an infection. There are numerous parasite species that are known to have taken advantage of these host behavioural changes. For example studies involving the cestode *Schistocephalus solidus* and its intermediate host the three-spined stickleback (*Gasterosteus aculeatus*) have shown that this parasite alters its host's behaviour in a way that facilitates transmission of the tapeworm to its definitive host, piscivorous birds. Behavioural changes included increased time spent foraging away from cover, reduced swimming performance and suppression of the host's anti-predator response (Barber *et al*., 2004).

Argulid parasite species are also known to cause behavioural changes in their fish hosts. Some of these appear to facilitate parasite transmission whereas others are clearly behaviours that have evolved as mechanisms employed by the host fish to try and dislodge lice from their external surfaces. Here we will discuss some of those changes documented by other researchers along with lice-induced behaviours witnessed by the authors here.

One of the first behavioural changes one can observe in fish harbouring an *Argulus* infection is the so-called flashing or scratching behaviour. Fish will repeatedly 'flash' their flanks against submerged objects such as plants, stones or even gravel. This behaviour is very common in fish infected with a wide range of ectoparasites and is believed to be an attempt at dislodging the culprits (e.g. *Gyrodactylus* spp., *Ichthyoptheirus multifilis* (white-spot), *Lernaea* spp.). Similarly, some fish leap clear of the water and certainly argulids will often detach from their hosts when exposed to the air. Communication between the authors and a commercial trout fishery in The Netherlands revealed that trout leaping clear of the water increases in frequency as water temperature increases and this coincides with an increase in the abundance of *A. foliaceus* within the lake (P. Walker *unpublished observations*). Sticklebacks have even been witnessed leaping onto the concrete surrounding of a garden pond in an attempt to rid themselves of a heavy lice infection. Sadly the efforts of these particular fish were thwarted by some resident frogs that viewed the floundering sticklebacks as an easy meal (Van der Velde pers com.). The determined effort by some fish to rid themselves of attached lice shows that lice are in fact extremely irritating to a fish's skin.

Other general behavioural changes associated with argulid infections include loss of appetite, denser shoaling behaviour, lethargy and changes in vertical positioning within a

water column. Our broodstock carp (*Cyprinus carpio*) harbouring *A. japonicus* were also observed to spend significant amounts of time crowding over air stones in their aquaria. This behaviour probably offers some relief to the irritation caused by lice living on the fishes bodies or it is even possible that the continuous bombardment by air bubbles eventually persuades the lice to dislodge from their hosts.

Research undertaken in Canada on the argulid species *Argulus canadensis* has revealed some interesting behaviour changes associated with *Argulus* infections. Dugatkin *et al*. (1994) showed that under experimental conditions juvenile sticklebacks will avoid schools of parasitized conspecifics even though the parasite itself did not elicit an avoidance response. Poulin (1999) showed that juvenile trout respond to the release of alarm substances emitted by conspecifics infected by *Diplostomum* parasites and it is possible that a similar event was occurring in the sticklebacks of Dugatkin's 1994 experiments.

Poulin and colleagues have shown some other behavioural changes associated with fish infected with argulids (e.g. Poulin and Fitzgerald, 1988). These researchers demonstrated that parasitic infections can have significant consequences on the community and population structures of fish. They revealed that in the presence of parasites fish will form larger, denser shoals. This strategy will decrease the chance that an individual will be targeted by parasites seeking a new host. However, the strategy also benefits the parasite by giving it an easy access to a wide range of potentially suitable hosts. Similar changes in shoaling behaviour were observed by Northcott *et al*. (1997) during an *Argulus* epizootic in a Scottish, Stillwater trout fishery. Sticklebacks were also noted to adjust their vertical positioning in the water column when infected with lice. Heavily infected fish would often just lie motionless for long periods on the bottom of ponds or experimental tanks.

5.2ii Damage to the host integument

Argulus spp. cause direct damage to their host’s integument through their attachment and feeding mechanisms (Chapters 7 and 8). This damage can result from either mechanical actions (i.e. from the sharp mouth parts) or from chemical secretions.

As mentioned earlier, the main attachment organs in adult lice are the maxillary suckers. In addition, various appendages are modified to form hooks or spines and microscopical examination of these structures clearly reveals how they may be damaging to the hosts integument. Whilst attached to their host argulids continuously beat their thoracopods back and forth to maintain a flow of fresh water over their bodies for respiratory

purposes. This results in pressure atrophy and small 'bruised' areas can often be observed when lice are removed.

The majority of the damage caused to the fishes skin results from the feeding activities of these parasites. The pre-oral stylet and labial spines have all been suggested as capable of secreting various toxins or digestive enzymes that facilitate the parasites feeding (Kearn, 2004 *and references therein*). These reported substances probably degenerate cells, making the mechanical feeding processes less strenuous for the lice.

The feeding action of argulids creates significant damage to the host integument. In chapter 8 we demonstrate that small craters can be formed in the host skin as a result of the feeding activities of these lice. Epidermal hyperplasia at the wound margins is also visible. Typically these craters are not much deeper than the epidermis but some examples of wounds reaching as deep as the stratum compactum have been recorded (Lester and Roubal, 1995). Mucous cells are generally absent within the craters themselves but a proliferation of these cells is frequently evident at wound margins (Lester and Roubal, 1995).

5.2iii Immune response

The immune response in mammals has been a topic addressed by many researchers throughout the 20th century. A strong focus has been on those pathogens that may ultimately have a significant implication for humans. This impact can take the form of disease symptoms in human populations directly or in the form of infections affecting livestock or domestic animals. With the increasing importance of fish for supplying protein to human populations researchers have begun to investigate those factors that may impact upon fish production i.e. pathogens. In order to combat these pathogens it is vital that we gain an understanding of the natural defence mechanisms employed by fish. This knowledge requirement has led to a new wave of research focusing upon the piscine immune system. In this section we will discuss some of those immune responses exhibited by fish that harbour parasites from the genus *Argulus*.

The immune system in fish can be described as having two parts, viz. i) the innate/non-specific immune response, and ii) the acquired/specific immune response. Recently there has been a renewed interest in the innate immune response of fish to pathogens because this consists of the first defensive 'barriers' that any foreign invader will encounter (e.g. epithelial barriers, acidic conditions in the gut, etc). These mechanisms are typically not pathogen-specific and similar infection types will illicit similar responses (i.e. localised inflammation, increase in mucous cell numbers, macrophage activation etc). The adaptive or

acquired immune response is so called due to its 'memory' properties. It normally takes several hours or even days for the animal to mount a response against a first infection from a pathogen but subsequent infections from the same pathogen will be met with an increasingly faster response. Typically, this involves the production of antibodies specific to certain antigen binding sites.

The immune response of fish to ectoparasitic organisms and particularly crustacean ectoparasites has only begun to receive special attention in the last decade and even then the research is rather unfocussed. It is, however, recognised now that this knowledge is of paramount importance if we ever hope to develop commercially viable and environmentally sound methods of treatment and control e.g. vaccines.

The most notable immune response to argulid infestations is observed as localised inflammation, which appears as small red spots on the fish's skin. The causative factor is often reported to be secretions from the pre-oral stylet of *Argulus* individuals but attachment of the parasite is also likely to have an impact on this response and certainly pressure atrophy has been documented previously (Lester and Roubal, 1995). The latter authors have suggested that secretions of the parasite have low antigenicity due to inflammation at feeding sites not being a major component of the histological changes. Ruane *et al*. (1995) demonstrated a humoral antibody response in rainbow trout (*Oncorhynchus mykiss*) after they had been immunised with an antigen extract from *A. foliaceus*. A similar type of response was seen in rainbow trout and Atlantic salmon (*Salmo salar*) after immunisation with sea lice antigens (Grayson *et al*., 1991; Reilly and Mulcahy, 1993). These data provide information that may prove invaluable for the development of vaccines against ectoparasitic crustaceans.

Inflammation is commonly associated with argulid infections and indeed large red spots can frequently be observed even within just a few hours post-infection (Chapters 7 and 8). The mechanisms involved in the inflammatory response have been well documented for mammals but considerably less information is available for fish. In a preliminary investigation of the early inflammatory immune response of carp to *A. japonicus* C. Haond and G. Wiegertjes (*pers. comm.*) measured the blood leukocyte redistribution over a period of 40 days post-infection. The relative percentage of granulocytes and monocytes, identified on the basis of typical forward/sideward scatter profiles in a flow cytometer, increased dramatically over time. The relative percentage of these phagocytic cell types was highest 30 days post-infection, when numbers of parasites on the skin also peaked, and declined thereafter with declining numbers of parasites. Skin samples from these infected fish were analysed for early (1 h post-infection) gene expression with RT-PCR, indicating increased

expression of the pro-inflammatory cytokines interleukin-1 and tumour necrosis factor alpha. These preliminary data support the existence of an inflammatory immune response to *Argulus*. Furthermore, Huising *et al*. (2003) analysing the same samples, demonstrated increased expression of particular CXC chemokines at the inflammation site, which could possibly explain the increase in granulocytes in the blood. Future work on the inflammatory response and other innate immune responses of fish is likely to show several comparisons with the mammalian systems, and as a result we may come to combine knowledge to develop successful control methods.

5.2iv Stress response

Cannon (1935) was one of the first researchers to introduce the concept of stress. He claimed that when the normal homeostasis of an organism was threatened by one or more stimuli that the organism could be considered as stressed. In 1992, Chrousos and Gold provided a more comprehensive definition of stress: "stress is a condition in which the dynamic equilibrium of animal organisms, called homeostasis, is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors". In this section we will focus upon the stress response of fish.

The physiological mechanisms of the teleost integrated stress response have been shown to share many similarities with that of terrestrial vertebrates (Wendelaar Bonga, 1997). The responses exhibited by a fish subjected to a stressor involve physiological and behavioural responses that are induced as mechanisms to try and protect homeostasis or maintain the dynamic equilibrium of the stressed organism (Wendelaar Bonga, 1997). These responses can be categorised as primary, secondary and tertiary responses (Wendelaar Bonga, 1997 *and references therein*).

The primary response involves dramatic increases in the blood levels of catecholamines and glucocorticoids. These hormones are the dominant hormones involved in the stress response and are the primary messengers of the two main routes through which the brain co-ordinates the stress response. These two routes include the hypothalamic-autonomic nervous system and the hypothalamic-pituitary-interrenal axis (Wendelaar Bonga, 1997). The secondary response comprises metabolic changes (i.e. plasma glucose and lactate levels), hydromineral disturbance (fluctuations in plasma chloride and sodium levels), haematological changes (e.g. in hematocrit and haemoglobin content) and changes in the hosts immune system (i.e. immunosuppression). Finally, the tertiary response consists of changes in the organism as a whole including reduced growth, impaired swimming performance, lower

reproductive success and reduced disease resistance, all of which can impact negatively upon the survival of the organism.

When examining the effects of a stressor, researchers typically use one or a combination of several parameters. Cortisol is the most widely used hormone involved in the stress response and because it is usually obtained from a fish's blood many researchers will also examine blood glucose levels in conjunction with cortisol and to a lesser extent serum sodium and chloride levels may be measured.

Other factors that can be indicative of stress include changes in the skin and gill epithelia. For example, skin from stressed fish exhibits abnormally high levels of apoptosis and necrosis which, if not fully compensated by cell proliferation, may result in epithelial disruption. In addition mucous cell discharge is stimulated and infiltration of the epithelia by leukocytes can be observed and indicates of the immune response is activated. Several effects of cortisol on the skin of rainbow trout (*Oncorhynchus mykiss*) *in vivo* have also been documented by Iger *et al.* (1995). Gill lamellae of stressed fish can appear irregular and swollen due to increased blood flow and blood pressure.

2.5.2v Parasites as stressors

Several studies have explored the hypothesis that sea lice infections elicit a stress response in fish (e.g. Nolan *et al*., 1999; Poole *et* al., 2000; Ruane *et al*., 2000). In contrast, very few studies have investigated the physiological effects of freshwater lice (*Argulus* spp.) on their hosts including whether they induce or affect the stress response of their hosts. For this reason we will include here the effects of sea lice as stressor.

Several authors have challenged fish to examine their stress response upon infection with ectoparasites. These studies have included a range of different host species and sizes but in general they have not been conducted using standard methods. What is clearly evident from many of these studies is that the level of stress caused by the infection is influenced by the intensity of the infection, host size, host condition and, possibly, host species of host (van Ham, 2003).

To enable some comparison between studies we have reported intensity of infection as the number of *L. salmonis* per gram of host body weight. In a study of sea lice, we examined the effects of ectoparasites in post-smolt Atlantic salmon, *Salmo salar* (Nolan *et al*., 1999). The direct effects of the parasite were the damage caused by parasite attachment and feeding on the body surface. The indirect stress effects included the effects on the overall integrity of the skin and gill epithelia such as increased apoptosis and necrosis of the superficially located

epithelial cells and decreased numbers of mucous cells in the skin. Reduced mucous cell numbers as a result of ectoparasitic infestations have also been reported in brown trout (*Salmo trutta*) epidermis (Pottinger *et* al., 1984). In the gills, where no lice were found, uplifting of the epithelium, intercellular swelling and infiltration by leukocytes is commonly observed in filaments and lamellae. High cell turnover of chloride cells was associated with significantly elevated gill Na^+/K^+-ATPase activities. These indirect stress effects are predominantly hormone mediated as a consequence of the parasite being perceived by the host, causing a stress response in the fish, and likely resulting in increased levels of blood cortisol and catecholamines.

Some of the differences between the stress response of terrestrial vertebrates and the stress response of teleosts can be attributed to the aquatic life style of fishes. The most important of these differences is the disturbance of the hydromineral balance in fishes, expressed by the changes in plasma sodium and chloride levels. Fish are directly exposed to the water over a large area via the epithelia covering the skin and gills. These epithelia are a complex assembly of many types of living cells (Whitear, 1986) and maintenance of epithelial integrity, particularly that of the gills, is essential for maintaining hydromineral balance, protection against waterborne pathogens, and thus ultimately the fish's health. High levels of catecholamines can influence the integrity of the branchial epithelium, probably by increasing the blood flow (and blood pressure) through the gills, and the permeability of the epithelium to water and ions (Wendelaar Bonga, 1997).

Nolan *et al*. (2000) examined the effects of low numbers of *Argulus foliaceus* on the epidermis of the rainbow trout. No effects were noticed on the number of mucocytes, but electron microscopic analysis of the upper cell layers revealed stimulated mucous cell discharge, and increased production of the small secretory vesicles of the pavement cells. These have been attributed antimicrobial activity, and the presence of peroxidase activity in these vesicles has been demonstrated. In addition to these signs of increased cellular activity, increased rates of apoptosis and necrosis were noticed. The intracellular spaces in the skin epithelium of the parasitized fish were enlarged and contained many leukocytes, most likely cells that had permeated the epithelium after leaving the blood system, since the number of circulating leucocytes was reduced 48 h post-infection.

In an attempt to distinguish between the direct effects of the parasites on the skin and the indirect effects mediated by hormonal messengers connected with the stress response, the effects of administration of cortisol (via the diet, to prevent the stress associated with injections) were studied (van der Salm *et al*., 2000). This showed that cortisol stimulates

mucous discharge, secretion of small vesicles by the pavement cells, apoptosis (but not necrosis) of epidermal cells, and leukocyte infiltration. Nolan *et al*. (2000) also noticed that low numbers of *A. foliaceus* (6 lice per fish) did not significantly elevate plasma cortisol levels 48 hrs post-infection. They attributed this to the fact that ectoparasites have co-evolved with their host organisms and therefore the host fish have evolved a tolerance to low numbers of lice (Nolan *et al*., 2000). Ruane *et al*. (1998) did, however, demonstrate elevated plasma cortisol levels in rainbow trout infected with *Argulus* 48 hrs after a 4 h confinement stress. This suggested that whilst the effect of lice may not be seen immediately after infection the effects become apparent when the response to a second stressor is examined (Nolan *et al*., 2000).

Infection with low numbers of the salmon louse *L. salmonis* generally does not result in significant increases in plasma cortisol levels of the host (Johnson and Albright, 1992b; Bjorn and Finstad, 1997; Ross *et al*., 2000). However, heavy infections may cause substantial elevation, far beyond those which cause immunosuppression (Johnson and Albright 1992b; Mustafa *et al*., 2000). Exposure of *O. mykiss* to juvenile stages of *L. salmonis* increased blood cortisol levels after 4 h net confinement to levels that were significantly higher than those in confined, but unparasitised fish (Ruane *et al*., 2000). Similar results were obtained with *O. mykiss* confined after 21 days of infestation with adult *A. foliaceus* (Ruane *et al*., 1999a).

The conclusions we can draw from these few studies include the fact that ectoparasitic lice can induce similar responses in fish skin to those stress responses observed for toxic stressors (Nolan *et al*., 2000). In addition, it was observed that certain fish species can tolerate low numbers of lice providing additional stressors are not encountered. This stress effect has implications for other host systems including the immune response and it is known that fish harbouring lice are frequently subjected to secondary infections possibly due to immunosuppression influenced by stress related responses (Bandilla *et al*., 2006). The next section highlights some of the more common secondary infections that fish infected with argulids may encounter.

5.2vi Secondary infections

In addition to the damage and stress caused by *Argulus* itself, one of the main concerns for fin fish producers is the associated secondary infections that can result from infections with parasites. Several studies have examined the role of parasites as vectors for other diseases (e.g. Nigrelli, 1950; Jones and Hine, 1983; Cusack and Cone, 1985; Cusack and Cone, 1986) and *Argulus* spp. have been the topic of some of these reviews (e.g. Dombrowski, 1952;

Ahne, 1985). The wounds created by this parasites feeding action are an obvious site for infection and bacterial e.g. *Aeromonas salmonicida*, Shimura *et al*. (1983) and fungal e.g. *Saprolegnia* spp. (Bower-Shore, 1940; Stammer, 1959) infections are often concurrent with *Argulus* spp. infections (Lester and Roubal, 1995). Some nematodes (e.g. Anguillicolidae and Skrjabillanidae) also use argulids as intermediate hosts (Moravec, 1978). The most worrying of these transmitted pathogens, however, is spring viraemia of carp (also known as infectious dropsy). This acute viral disease of carp kills hundreds of wild and captive carp every year. Argulids have long been suspected as vectors for this virus (Dombrowski, 1952) but it was only in the 1980's that this was proved satisfactorily through controlled laboratory experiments (Ahne, 1985).

6. Control and prevention

There are numerous options available for the control, prevention and treatment of *Argulus* infections. Much of the literature recognises that Argulosis, like many other diseases, is best defended against by good fish husbandry and stock management. Quarantining fish is vital for the aquarist but not always feasible for large-scale fish production (e.g. trout farming). Individual fish can then be examined for the presence of parasites and any encountered can be carefully removed using forceps (Benz *et al*., 2001). Care must be taken to examine buccal and gill cavities also because on occasion *Argulus* individuals have been found there. Large scale fish production requires other control methods suitable for treating large numbers of fish.

There are numerous options available for the control, prevention and treatment of *Argulus* infections. Many traditional treatment methods rely on the use of toxic chemicals such as malachite green or other insecticide type chemicals. The damaging effects of many of these chemicals on wildlife are now widely recognized and as a result many countries now prohibit their use. There is also an increasing demand from consumers for food fish that have not been subjected to chemical treatments. The wide range of chemical treatments available for infections of *Argulus* spp. are well documented. Due to the extensive literature available on this topic no effort is made here to discuss them in detail. The author therefore refers the reader to selected texts, e.g. Kabata (1970, 1985), Williams (1997), Van Duijn Jnr (1973) and Lester and Roubal (1995).

In addition to the chemical treatments available, scientists are intensively examining new methods that may prove cost-effective and, more importantly today, environmentally

friendly. The use of invertebrate developmental inhibitors (IDI's) is now under review for the treatment of fish ectoparasites. For example, Williams (1997) recently examined the effectiveness of a chitin inhibiting treatment (diflubenzuron) for the treatment of *Argulus* infestations and particularly the success from oral administration of these chemicals. Whilst the results showed some effect against the parasite the study was not conclusive. Combining the drug with the fish feed in a way that proved palatable for the fish and viable as a treatment proved to be the main difficulty.

During the last couple of decades researchers seeking cost-effective, environmentally friendly methods to control crustacean ectoparasites have come up with some interesting biological control methods. In wild situations many fish utilize cleaner fish (commonly various species of wrasse) to help rid themselves of parasites. In Norway, fish farmers have employed wrasse in salmon cages to help control ectoparasitic copepod numbers (Bjordal, 1991). This method has shown some promise but has not been universally successful. In 2002, Gualt *et al*., published a paper detailing the use of novel egg-laying boards to control argulid numbers in a commercial, stillwater trout fishery. Boards were positioned in the water column with the idea that lice would use them as sites for egg deposition. The results were very promising therefore further investigations are being undertaken by this research group.

7. Conclusions

Argulus spp. rarely have significant impacts upon natural fish populations. Epizootics are observed when the natural equilibrium is perturbed by one or more factors and in many cases anthropogenic actions have been implicated. For example, increased population densities in fish farms and even commercial sport fisheries facilitates parasite transmission and stress resulting from crowding, capture, handling and confinement can have a deleterious effect upon the fish's immune response. Menezes *et al. (*1990) provided evidence that stocking water bodies with non-native fish species can also provide easy targets for lice and, as we have seen with *A. japonicus*, anthropogenic transfer of fish can also facilitate parasite dispersal (Rushton-Mellor, 1992).

Much of the research with argulids in the past has focused on morphological and ecological aspects of these organisms. Whilst this information is useful in understanding certain aspects of the parasites life cycle and mode of existence the more recent research focusing on the host-parasite interactions shows considerable promise in helping to develop economically viable and environmentally sound methods of controlling these parasites. If the

fish and lice have co-evolved over many generations it seems logical that we should consider both organisms together when trying to solve the problems we face.

With continuing advances in the field of fish immunology future research is likely to increase our knowledge of the intimate relationship between argulids and their fish hosts. Many of the advances in sea lice research may also contribute to our understanding of the relationship between freshwater lice and their hosts. In addition to increasing our knowledge of fish defense mechanisms and the host parasite interactions, we must also consider the parasite itself. Much of the basic knowledge regarding these organisms biology is still not fully understood and in many cases may prove vital to our insight into the complex relationships between pathogens and their hosts.

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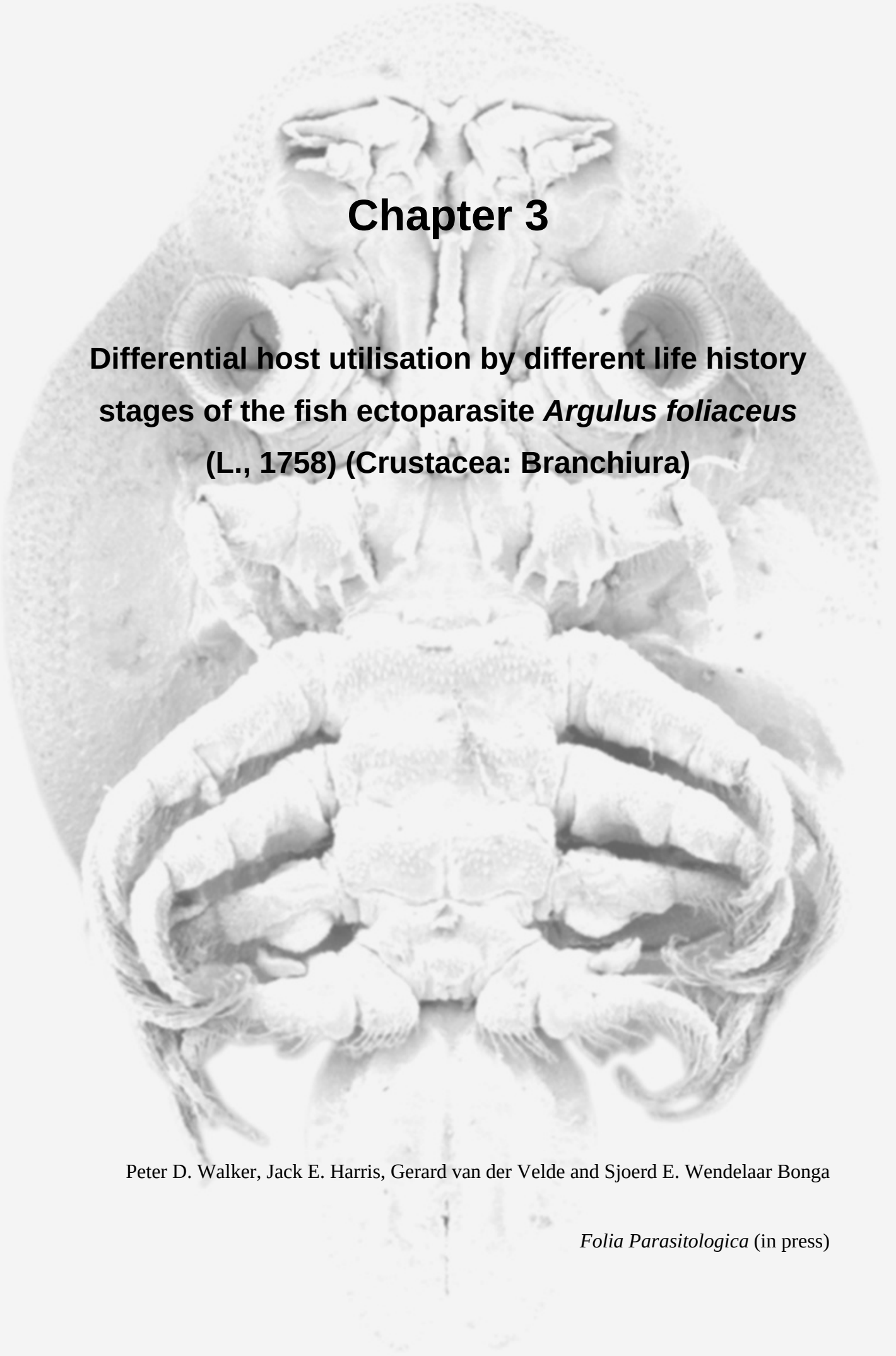

Chapter 3

Differential host utilisation by different life history stages of the fish ectoparasite *Argulus foliaceus* (L., 1758) (Crustacea: Branchiura)

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Folia Parasitologica (in press)

Abstract

In this study we examine differences in the occurrence of life history stages of the destructive fish ectoparasite *Argulus foliaceus* (L., 1758) on eight fish species (stickleback, rudd, roach, gudgeon, bream, tench, crucian carp and common carp) sampled from a mixed-species recreational fishing lake on nine occasions during late spring and summer. Total numbers of *A. foliaceus*, as well as the number of larval, juvenile and adult parasite stages, from each fish were recorded along with the fish species. Lice generally exhibited an aggregated distribution approximating a negative binomial distribution. Significant differences in the prevalence, intensity and intensity frequency distribution were observed between life history stages and between host species. In general, all life history stages of *A. foliaceus* exhibited an over-dispersed disribution. However, larval lice did show some degree of aggregation, particularly within the stickleback samples. Infection data for parasite larval stages suggested that sticklebacks are more likely to be infected than other host species. For adult lice, however, carp appeared to be the main host. We propose that *A. foliaceus* infection characteristics are predominantly determined by the level of host exposure to the parasite and its life history stages (larval, juvenile and adult), rather than by an innate difference in susceptibility related to individual host factors such as immune responses. We conclude that host exposure is determined by the parasite-host behavioural interplay related to species specific ecology and behavioural traits such as microhabitat preference and normal swimming speed.

Introduction

The ecology of freshwater macrophytes, planktonic organisms and macro-invertebrates is well described but many common parasites are still poorly studied. There is a plethora of published material relating to fish parasites (e.g. Kabata, 1985; Barber *et al*., 2000; Nolan *et al*., 2000; Benz *et al*., 2001). Many of these papers have focussed upon cultured, and laboratory-reared host-parasite models and, in some cases, data regarding the natural ecology and infection dynamics of are considerably lacking by comparison.

Host-specificity has always been an area of interest for parasitologists as many parasite species show high levels of specificity, typically being restricted to just one species or genus (Roberts and Janovy, 1996). Intermittent parasites appear to be an exception to this trend with several groups exhibiting relatively low specificity, e.g., many lice, fleas, leeches, mosquitos and midges. The fish louse *Argulus foliaceus* (L., 1758) seems to share this non-host-specific trait with other intermittent parasites as occurs on a wide range of freshwater fish species (Gurney, 1948, Kollatsch, 1959, Stammer, 1959, Kennedy, 1974, Lester and Roubal, 1995, Holland and Kennedy, 1997, Kearn, 2004, Walker *et al*., 2004).

Many authors have commented on the lack of specificity of argulid parasites, sharing the opinion that individual species from this group (e.g., *A. foliaceus* and *A. japonicus* Thiele, 1900) can infect a wide range of host species (Kearn, 2004 *and references therein*; Walker *et al*., 2004 *and references therein*). However, some apparent host preferences have been demonstrated (e.g., Valtonen *et al*., 1997, Mikheev *et al*., 1998, 2000, Pasternak *et al*., 2000).

Most publications regarding host preferences of branchiurans concentrate on infection dynamics within just one or two host species (e.g., LaMarre and Cochran, 1992, Mikheev *et al*., 1998, Pasternak *et al.*, 2000), often in non-natural situations such as laboratory aquaria or fish farms (e.g., Hakalahti and Valtonen, 2003, Mikheev *et al*., 2004). There is a noticeable lack of published information regarding the distribution and epidemiology of *A. foliaceus* in mixed species fish communities.

It is probable that, for behavioural and ecological reasons, some fish species are more likely to be infected by non-host-specific parasites than others. Bandilla *et al.*, (2005) suggested that host-behaviour leading to increased exposure to lice was the most likely mechanism leading to observed infection characteristics of *Argulus coregoni* Thorell on rainbow trout, *Oncorhynchus mykiss* (Walbaum), from a Finnish fish farm.

On this basis we hypothesise that certain fish species are more likely to be infected with different life history stages of the non-host-specific ectoparasite, *A. foliaceus*, due to the increased likelihood of encounters related to species specific ecological and behavioural traits.

This study aims to use field observations of infection characteristics of *A. foliaceus* on several host fish species to highlight the importance of considering parasite ontogeny when looking at parasite distributions amongst hosts and also the importance of considering all members of a fish community when studying such an apparently non-host-specific parasite.

Materials and methods

Fish sampling

Sampling took place in one lake of a mixed species, freshwater, commercial fishery in South West England (OS grid reference: SX456751). The lake has a surface area of approximately 2 acres and an average depth of approximately 90cm. This man-made fishing lake was completed and opened to the angling public in 1995. Fish were sampled on nine occasions during the late spring and summer season, a period when *A. foliaceus* is known to be most abundant and when all life history stages are present (Walker *et al.*, 2004). Fish were mostly sampled with angling techniques; samples of small fish inhabiting the dense weed beds of the shallow littoral zone were obtained with a standard pond net. During each sampling trip several different areas were fished and within each area anglers were fishing at several depths and locations during the course of the day. Standard angling methods were used for several reasons: i) the fishery owner wished it; ii) the authors felt this method of fish capture causes the least disturbance to the environment; iii) it did not cause excessive damage to the fins, scales, mucous layer and epidermis of the fish; iv) it reduced the risk of parasites being 'rubbed' off during capture; v) several regions of the fishery and several species of fish were targeted simultaneously by using several anglers. Upon capture all fish were placed on a pre-wetted, white mat (to aid spotting of dislodged parasites) and handled with wet hands to minimise damage to the epithelium and the protective mucous layer. The eyes of larger fish were covered with a damp cloth to calm the fish during parasite collection.

Parasite collection

The external surfaces and buccal and gill cavities of all fish were examined thoroughly for *Argulus* individuals which were subsequently removed carefully using a set of blunt forceps. The total number of parasites (referred to as “all lice” from here on) and the number from each life history stage (larval, juvenile and adult) collected from the fish was recorded along with the fish species they were taken from. Parasite developmental stage was classified as larval, juvenile or adult according to descriptions by Rushton-Mellor and Boxshall (1994).

Data analysis

Parasite numbers from all nine sampling occasions were pooled to give an overview of the infection characteristics during the sampling period. Statistics were calculated using methods described by Rózsa *et al*. (2000) with Quantitative Parasitology 3.0 (QP 3.0; Reiczigel and Rózsa, 2005). Descriptive statistics include prevalence (proportion of infected hosts amongst all hosts examined) with better exact confidence limits and mean intensity (average number of parasites found on the infected hosts – zeros of uninfected hosts were excluded) with bootstrap confidence limits (BCa) as recommended by Rózsa *et al.* (2000). As measures of aggregation we calculated the Index of Discrepancy (D) as described by Poulin (1993) and tested the infection intensity frequency distribution to see how closely it fits a negative binomial distribution (K). All confidence intervals are given at the 95% level unless otherwise stated.

Differences between host species numbers were analysed with ANOVA and Dunns post-hoc test using INSTAT. Chi-square test was used to test differences between prevalences as recommended by Rózsa *et al.* (2000). Bootstrap 2-sample t-test was applied to test for differences between mean intensities. This test is considered more appropriate for parasites due to the skewness of their distribution (Rózsa *et al*., 2000). A Brunner-Munzel test of stochastic equality was applied to test differences between parasite intensity distributions (Reiczigel *et al*., 2005)

Results

Fish species

Eight species of fishes were encountered during the sample period: three-spined stickleback *Gasterosteus aculeatus aculeatus* L.; rudd *Scardinius erythropthalmus* (L.); roach *Rutilus rutilus* (L.); gudgeon *Gobio gobio gobio* (L.); common bream *Abramis brama* (L.); tench *Tinca tinca* (L.); crucian carp *Carassius carassius* (L.); common carp *Cyprinus carpio carpio* L.. Not all species were equally represented in samples (Table 1) indicating that there are probably greater numbers of some species than others within the resident fish community. Rudd (24%), carp (22%), gudgeon (18%) and roach (13%) were the most abundant species in samples (Table 1).

Table 1. The proportion of the sampled fish community made up by each species and the distribution of all lice (larval + juvenile + adult stages), larval lice, juvenile lice and adult lice on different host species.

Fish species	Proportion (%)				
	Total fish com.	**All lice**	**Larval lice**	**Juvenile lice**	**Adult lice**
Stickleback	8	32	83	26	9
Rudd	24	4	0	9	2
Roach	13	< 1	0	1	0
Gudgeon	18	18	10	19	22
Bream	6	6	0	5	11
Tench	4	2	0	2	4
Crucian carp	5	1	1	3	< 1
Common carp	22	37	6	35	52

Infection data

Fish lice (*Argulus foliaceus*) were found on all sampling days (identified with characteristics described by Fryer (1982)). Of the 650 fish sampled, 241 were infected with *A. foliaceus*. Infection summary statistics for all, larval, juvenile and adult lice are summarized in Table 2. All the louse populations show infection intensity frequencies that exhibit an over-dispersed pattern with many fish harbouring low numbers and nly a few fish with high numbers of lice. These data fit a negative binomial distribution in all cases (Fig. 1).

Water temperature during the sampling period ranged from a minimum of 15.5^{o}C at the start of the sampling period to a maximum of 22.5^{o}C at the end of the sampling period. The abundance of all lice and the different life history stages on the different fish species varied during the course of the sampling period (Fig. 2A-D). However, a general trend is apparent with larval and juvenile stages typically being more abundant on sticklebacks than other species throughout the sampling period (Fig. 2B, C). Adult lice, however, were consistently more abundant on carp and gudgeon with sticklebacks sometimes appearing as an important host species (Fig. 2D). From the beginning of June until the end of the sampling period, larval lice exhibit a much greater abundance than adult lice and this pattern was similar for juvenile stages although their numbers did not increase until mid to late June. The abundance of all lice increased during the first half of the sampling period and then appears to level off and even decrease towards the end of the sampling period. The abundance of larval and juvenile stages match this pattern quite closely, however, the abundance of adult lice appears to remain relatively stable throughout the sampling period.

Prevalence is highest for adult lice followed by juvenile and then larval lice. The prevalences of the different life history stages differ significantly (Chi-square = 112.75, 2df; P <0.0001). Lower average infection intensity typically coincided with higher prevalence. Mean infection intensity in larval lice was significantly higher than in adult (t = -4.22, 2-sided P-value <0.01) and juvenile stages (t = -3.85, 2-sided P-value <0.01). Infection intensity for juvenile lice was also significantly higher than for adult lice (t = -2.18, 2-sided P-value <0.05). Infection intensity distributions of adult and larval stages and juvenile and larval stages also differed significantly (2-tailed P-values <0.00001 and 0.0001, respectively) but not between adult and juvenile stages (2-tailed P-value = 0.177).

Table 2. Mean and maximum intensity (I), prevalence and Index of Discrepancy (D) (after Poulin 1993), for all lice , larval, juvenile and adult lice are shown for the whole fish community and the stickleback, rudd, gudgeon and carp populations. Data for roach are not given due to the very low louse prevalence on this species. The 95% confidence limits for each value are shown in parentheses.

Parasite group	Parameter	All fish (n = 650)	Stickleback (n = 53)	Rudd (n = 153)	Gudgeon (n = 119)	Carp (n = 140)
All lice	**Prevalence**	37.1 (33-41)	56.6 (42-70)	8.5 (5-14)	57.1 (48-66)	65.7 (58-73)
	Mean I	2.8 (2.5-3.3)	7.3 (5.4-9.6)	2 (1.5-2.5)	1.8 (1.5-2.2)	2.7 (2.3-3.1)
	Max I	23	23	4	8	9
	D	0.796	0.667	0.932	0.601	0.587
Larval	**Prevalence**	3.8 (2.6-5.7)	28.3 (18-42)	N/A	3.4 (1-8)	2.9 (1-7)
	Mean I	6.4 (4.6-8.4)	8.9 (6.6-12.1)	N/A	4.0 (1.3-6.3)	2.0 (1.0-3.3)
	Max I	22	22	N/A	7	4
	D	0.977	0.796	N/A	0.970	0.973
Juvenile	**Prevalence**	17.4 (14.6-20.5)	24.5 (15-38)	7.2 (4-13)	24.4 (17-33)	28.6 (22-37)
	Mean I	2.1 (1.9-2.4)	4.8 (3.6-5.5)	1.9 (1.3-2.6)	1.6 (1.3-2.0)	2.2 (1.7-2.7)
	Max I	7	7	4	5	7
	D	0.888	0.789	0.944	0.81	0.810
Adult	**Prevalence**	24.8 (21.5-28.2)	30.2 (19-44)	2.6 (1-6)	39.5 (31-48)	50 (42-58)
	Mean I	1.8 (1.6-1.9)	1.5 (1.2-1.9)	1.3 (1.0-1.5)	1.3 (1.2-1.4)	2.1 (1.8-2.5)
	Max I	7	3	2	2	7
	D	0.825	0.735	0.971	0.663	0.662

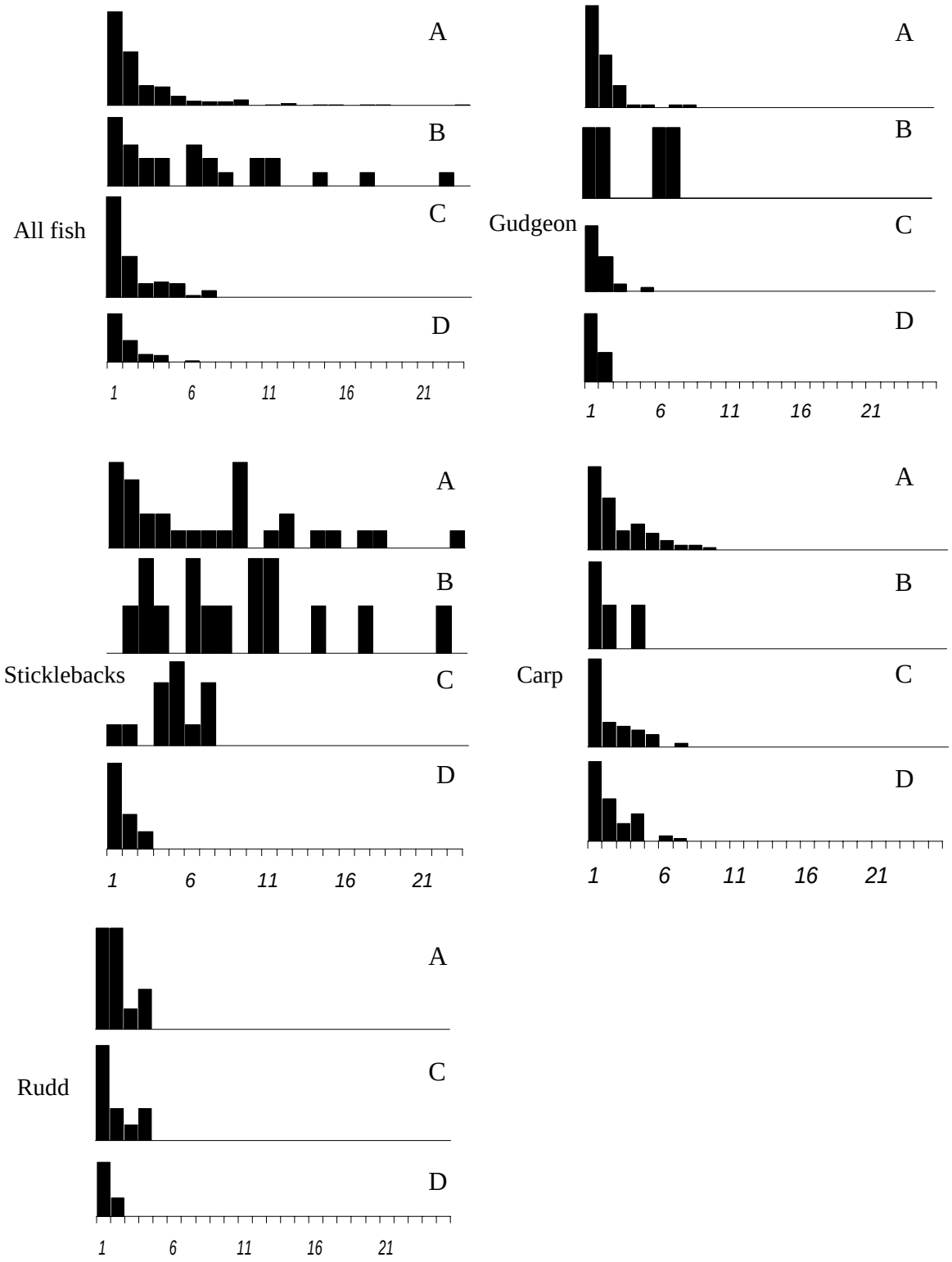

Fig 1. Intensity frequency histograms for all lice (A), larval (B), juvenile (C) and adult stages (D) infecting different fish groups.

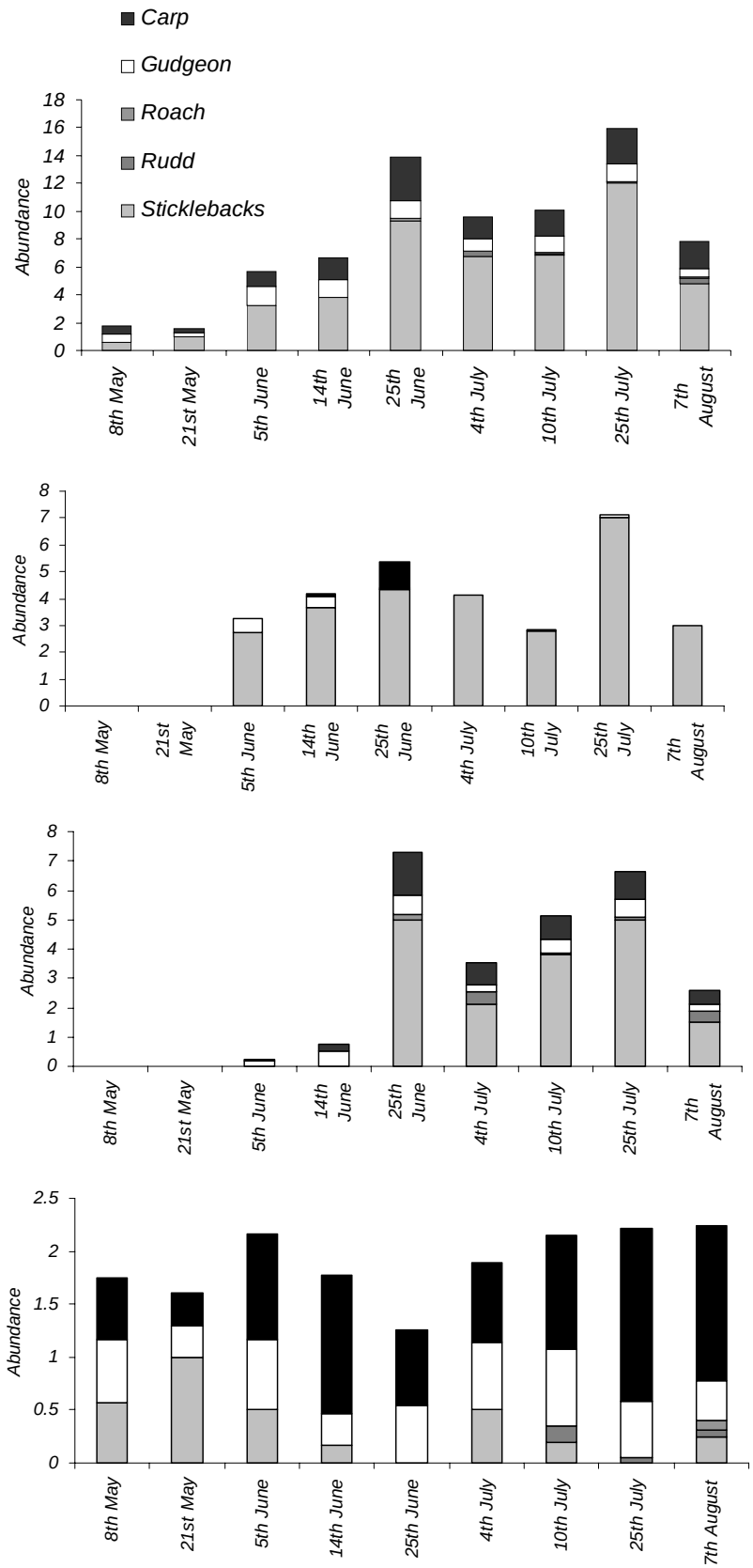

Fig. 2. Stacked bar charts showing the abundance (number of lice/number of fish sampled) of all lice (A), larval (B), juvenile (C) and adult stages (D) on five different fish species sampled on nine occasions.

Lice were not distributed evenly between the fish species and the distribution of larval, juvenile and adult stages showed differences in terms of their occurrence on different host species (Table 1). The majority of the lice sampled were found on carp and sticklebacks with a large proportion also being found on gudgeon (Table 1). Relatively few lice were observed on the other fish species caught. Larval lice were found predominantly on sticklebacks. Juvenile louse distribution approximates closely that of all lice together, with carp and sticklebacks harbouring the majority of juvenile lice followed by gudgeon and again relatively few lice being found on the other host species (Table 1). Adult lice, however, were found predominantly on carp followed by gudgeon and then bream. Tench, crucian carp and bream samples were not subjected to further statistical analyses due to the low number of individuals sampled.

Infection data for the stickleback population

Of the 53 sticklebacks examined, 58% were infected with *A. foliaceus* (Table 2). The whole louse population and the larval lice portion, fit the negative binomial distribution (Fig. 1). The juvenile and adult lice portions do not fit the negative binomial distribution. Lice infecting the stickleback population appear to be more aggregated in their distribution than the pattern for the whole fish community.

Prevalence ranged from the highest for adult lice to the lowest for juvenile lice. However, prevalences were not significantly different (Chi-square = 0.44, 2df; P = 0.803). The mean infection intensity of larval lice was significantly higher than the mean infection intensity of juvenile and adult lice ($t = -2.723$, 2-sided P-value <0.05 and $t = -5.113$, 2-sided P-value <0.001, respectively). The mean intensity of juvenile lice was significantly higher than that of adult lice ($t = -6.053$, 2-sided P-value <0.0001).

Aggregation was highest in larval lice followed by juvenile and then adult stages (Table 2). Infection intensity distributions of the three life history stages differed significantly in all cases (2-tailed P-values: adults versus larvae, $P <0.001$; adults versus juveniles, $P <0.001$; juveniles versus larvae, $P <0.05$).

Infection data for the roach population

Prevalence was very low (3.5%, $0.001 < 95\%$ cl < 0.1) with only three out of 85 fish harbouring lice. Two fish harboured one juvenile louse each and the third fish harboured one adult louse. Due to the extremely low numbers of lice statistical analyses were not possible.

Infection data for the rudd population

Of the 153 rudd examined, only 8.5% were infected with *A. foliaceus* (Table 2). The whole louse population and the juvenile portion fit a negative binomial distribution (Fig. 1). No larval lice were found on rudd and there were insufficient adult lice to test the intensity frequency distribution. Infection intensities were considered low in all cases (maximum of 4 lice per fish).

Prevalence of the different life history stages differed significantly (Chi-square = 12.819, 2df; P <0.005) with juveniles exhibiting the highest prevalence. However, the prevalences were considered low (<8%) in all cases. Juvenile louse mean infection intensity was higher than that of adults (Table 2) but the difference was not significant (P >0.05).

Infection data for the gudgeon population

More than half of the gudgeon sampled were infected with *A. foliaceus* (Table 2). In the case of all lice, larval and juvenile stages, the infection intensity frequency data fit a negative binomial distribution (Fig. 1).

Adult prevalence was highest followed by juvenile and then larval lice (Table 2). Prevalence of the different life history stages differed very significantly (Chi-square = 45.067, 2df; P <0.0001). Larval lice exhibited the highest mean infection intensity followed by juvenile and then adult lice. However, mean intensities of the different life history stages did not differ significantly in all cases. Larval lice also exhibited the highest degree of aggregation, again followed by juvenile and adult stages (Table 2) but intensity distributions did not differ significantly.

Infection data for the carp population

Of the 140 carp sampled, 66% were infected with lice (Table 2). The intensity frequency distribution of the louse population as a whole fit a negative binomial distribution as did the juvenile louse portion (Fig. 1). Adult louse intensity frequency distribution did not fit a negative binomial distribution, however, and there were not enough categories of larval lice to test the fit.

Adult lice exhibited the highest prevalence followed by juvenile and then larval lice (Table 2). Prevalence of the different life history stages differed significantly (Chi-square = 78.885, 2df; P <0.0001). Mean infection intensities of the three life history stages were all very similar (Table 2) and did not differ significantly. Larval lice exhibited the highest degree

of aggregation followed by juveniles and then adults (Table 2). However, the infection intensity distributions did not differ significantly.

Between species comparisons

The prevalence of all lice and of larval, juvenile and adult life history stages differed significantly between species in all cases (Chi-square = 172.242, 4df; P <0.0001). The infection intensity of all lice on sticklebacks was significantly higher than all other species (2-sided P-values <0.01 in all cases). Mean infection intensity on roach was significantly lower than those of all other species (2-sided P-values <0.03 in all cases). Mean infection intensity on rudd was significantly higher than roach (2-sided P-value <0.03) but not significantly different from gudgeon (2-sided P-value >0.1). The mean infection intensity of carp was higher than that of rudd although the difference was only marginally significant (2-sided P-value = 0.085). Mean infection intensity on gudgeon was significantly lower than on sticklebacks and carp (2-sided P-values <0.03) but not significantly different from rudd (2-sided P-values >0.05).

Only sticklebacks, gudgeon and carp harboured larval lice. Mean infection intensity of larval lice on sticklebacks was significantly higher than on gudgeon or carp (2-sided P-values <0.05 respectively). Mean infection intensity of larval lice on gudgeon was higher than that of carp (Table 2) but the difference was not significant (2-sided P-value >0.05).

All five species were infected with juvenile lice. However, only two roach harboured juvenile lice, therefore this species was not included in the statistical analysis. Juvenile louse infection intensity was significantly higher on sticklebacks than on all other host species (2-sided P-values <0.05 respectively). Juvenile louse infection intensity on rudd was slightly higher than on gudgeon and slightly lower than on carp but the differences were not significant (2-sided P-values >0.05 respectively). Infection intensity of juvenile lice on carp was slightly higher than on gudgeon and the difference was considered marginally significant (2-sided P-value = 0.06).

For adult louse infection intensity, roach were again excluded from statistical analysis as there was only one fish harbouring adult lice. Adult louse mean infection intensity on carp was significantly higher than on sticklebacks, rudd and gudgeon (2-sided P-values <0.05 respectively). Mean infection intensity of adult lice on sticklebacks was slightly higher than on rudd or gudgeon but the differences were not significant (2-sided P-values >0.05). Infection intensities of adult lice on rudd and gudgeon also did not differ (2-sided P-value >0.05).

We also tested the intensity distributions of lice, and of the different life history stages, between the different species. Roach were excluded from all analyses, except that of all lice, due to the low number of infected fish for this species. In addition, rudd were excluded from analyses of larval lice because no rudd were found with this parasite life history stage. Intensity distributions of all lice were significantly different between all species (2-sided P-values <0.05) except between rudd and gudgeon, and rudd and carp (2-sided P-values >0.05). The difference between larval lice intensity distribution on sticklebacks and carp was significant (2-sided P-value <0.05), between gudgeon and sticklebacks the difference was marginally significant (2-sided P-value = 0.06) and the difference between carp and gudgeon was not significant (2-sided P-value >0.05).

Juvenile lice intensity distributions on sticklebacks differed significantly from rudd, gudgeon and carp (2-sided P-values <0.01 in all cases). Intensity distributions on rudd did not differ significantly from gudgeon or carp (2-sided P-values >0.05) and intensity distributions did not differ between carp and gudgeon either. Finally, intensity distributions of adult lice differed significantly between carp and gudgeon (2-sided P-value <0.01) but not between any of the other species.

Discussion

A range of different regions and depths around the lake were sampled at several different times during the day and on each sampling trip several angling methods were employed to try and ensure that any bias in fish species caught could not be attributed to sampling locality or sampling technique. Very heavily infected fish are likely to be lethargic and spend less time feeding than lightly infected or uninfected individuals (Wendelaar Bonga, 1997, Walker *et al.*, 2004), which would reduce the likelihood of these individuals being caught. No moribund or dead fish were observed during the whole of the sampling period and the fishery owner had not reported such occurrences. Infection levels were generally not high enough to cause serious effects, e.g., morbidity and lethargy, associated with epizootics. Lice could be dislodged during capture of the fish, but Bower-Shore (1940) observed that a fast flowing stream of water in the laboratory did not dislodge adult parasites. Therefore, the chosen sampling methodology probably did not result in significant losses of lice during fish capture. No data is available to determine if the attachment strength differs between life history stages. An alternative sampling technique that would exclude all of the above possible biases or provided more reliable data is currently unavailable.

The abundance data hint towards a trend of certain fish species being favoured as hosts by different life history stages of *Argulus foliaceus*. The relative abundance of larvae and juveniles on the different host species does not appear to vary greatly throughout the sampling period although their numbers do vary considerably. However, the abundance of adults on sticklebacks does vary noticeably throughout the sampling period. Adult lice appear to be more abundant on sticklebacks approximately 4 weeks prior to the first big peak in larval lice numbers and approximately 3 weeks prior to the second big peak in larval lice numbers. Our own laboratory observations have shown that *A. foliaceus* eggs can hatch in only 18 days at 20^{o}C and 28 days at 15^{o}C (unpublished data). Therefore, it appears that adult lice may utilize sticklebacks as a temporary host whilst they move into shallow water to deposit their eggs which subsequently hatch after 2-6 weeks (depending on temperature) resulting in the observed peaks in larval lice abundance that we observed.

If *A. foliaceus* and the various host fish species were evenly distributed with the water body, parasite distribution should be related to the number of available hosts from each species. However, in terms of total numbers of lice, parasite distribution amongst the host community does not appear to be related to the number of available hosts from each fish species in our study.

All fish species sampled are known to be potential hosts for *A. foliaceus* (Kennedy, 1974). The infection data for the fish community as a whole showed that adult louse prevalence (i.e., proportion of the community harbouring adult lice) was significantly higher than both juvenile and larval louse prevalence. In contrast larval lice show the highest mean intensity and adult lice the lowest mean intensity. This indicates a trend of larval lice having a more aggregated distribution than other life history stages but becoming more dispersed as they mature. Eggs of *A. foliaceus* are deposited in clumps or, more commonly, parallel rows with as many as several hundred eggs in a clutch (Mikheev *et al.*, 2003; Kearn, 2004; Walker *et al*., 2004). A firm substrate such as a plant stem or surface of a stone, is required for egg deposition (Kearn, 2004), and therefore certain areas where a large surface area of suitable substrate is available may become 'hot-spots' for infection of fish with larval lice leading to an aggregated distribution on hosts that occur in these regions. The intensity frequency data support this (Fig. 1), showing that several individuals were infected with relatively high numbers of larval lice (up to a maximum of 22 lice) and very few fish with more than 4 adult lice.

As a generalist parasite, *A. foliaceus* probably does not actively discriminate between fish species. However, if certain regions are indeed hot-spots for infection with larval lice

then fish that frequent these regions are more likely to become infected with larval lice than fish that generally occur in other regions of the water body. Mikheev *et al.*, (2003) suggested that most freshwater fishes concentrate close to the shore in late spring and early summer for spawning and foraging thereby increasing the risk of exposure at least to larval and adult female parasites. Urho (1996) showed that *A. foliaceus* occurs almost exclusively in the littoral zone of a lake during the summer although no distinction was made as to what life history stage was encountered. We found that adult *A. foliaceus* was more common in open water habitats (data not shown) but it should be noted that our study was conducted in a very small, shallow (mean depth < 1m) pond the whole of which is probably representative of a larger lake's littoral zone.

Prevalence of the three parasite development stages on sticklebacks did not differ significantly. However, mean infection intensities did, with larval lice showing much higher intensities than adult and juvenile stages, and adults showing the lowest. Sticklebacks inhabit the shallower, more sheltered regions of stillwaters (Davies *et al*., 2004), particularly during the spring and summer periods for reproduction purposes and because of higher food abundance (Mikheev *et al.*, 2003, Davies *et al*., 2004). These sheltered, littoral regions (e.g., reed beds and pier supports) are also the areas generally chosen for egg deposition by adult female *A. foliaceus* (Walker *et al*., 2004). This would place sticklebacks at high risk of infection by larval lice and the present data and those of Walker *et al*., (2007) support this.

In aquaria *A. foliaceus* showed a preference for juvenile roach over perch under light conditions, with the reverse being found under dark conditions (Mikheev *et al*., 1998). In the field, perch exhibited higher infection levels than roach (Valtonen *et al.*, 1997). In our study we only encountered three roach harbouring lice, only one of which was an adult louse. Mikheev *et al.* (2000) later demonstrated that *A. foliaceus* also changes its hunting strategies depending on whether conditions are light or dark. The small lake sampled for our study was very turbid and light intensities were probably quite low only several cm below the surface. This is typical of lakes containing many carp due to their feeding habits (Davies *et al*., 2004). Herter (1927) suggested that *A. foliaceus* avoids the surface zones with too much light and this could be to avoid the 'sensory overload' mentioned by Mikheev *et al*. (2003). This hypothesis was deduced from lice in glass aquaria. However, rippling on the water surface may cause light reflections resulting in similar confusing effects for the lice. This effect may cause behavioural changes in the lice resulting in avoidance of this zone. If this behavioural change does occur then lice will be subjected to lower light intensities resulting from high turbidity and this could influence *A. foliaceus* to adopt the 'dark-hunting-strategy', which may

not favour the location of roach as suggested by Mikheev *et al*., (1998). In addition, roach tend to show a fairly ubiquitous distribution within stillwaters with no obvious microhabitat preference (Davies *et al*., 2004). Larval lice especially are more likely to occur in regions with vegetation, or around other structures more common in shallow littoral zones, due to the egg deposition habits of adult female lice. Juvenile and adult stages may be more dispersed if lower light intensities do encourage a shift towards the 'dark-hunting-strategy'. Then fish that are swimming in close proximity to the lake bottom and which are not highly active in their behavioural habits (e.g., gudgeon, carp and stickleback) are more likely to become infected than those showing more pelagic or surface-dwelling habit (e.g., roach and rudd).

Whilst the prevalence of *A. foliaceus* on rudd is more than double of that on roach, at 8.5% it is still a very small portion of the population that is infected. Rudd tend to swim close to the surface in proximity to, but not necessarily within, vegetation stands (Davies *et al*., 2004). As we stated earlier, larval lice are more likely to be found within vegetation stands rather than outside of them and all lice are likely to be more common near the bottom (Herter, 1927) in a different vertical zone to that typically inhabited by the rudd. Rudd are relatively active, fast swimmers when compared with species such as gudgeon and sticklebacks. The relatively slow swimming speed of *A. foliaceus* (especially the larval stage) may mean that rudd provide a difficult target for lice to locate and successfully attach to.

More than half of the gudgeon population was infected with lice. Gudgeon are benthic feeders (Davies *et al*., 2004), so are more likely to encounter lice showing the 'dark-hunting-strategy' and swimming closer to the bottom away from the bright, reflective, surface zones. Gudgeon are a slow swimming, shoaling species often occurring in large shoals which would facilitate parasite transmission by dispersing them amongst the population. Thus, we would expect a high prevalence but not necessarily a high intensity, and this is indeed what we observed. The higher intensity of larval lice is concurrent with a low prevalence suggesting that a small number of fish had encountered a hot-spot where larval lice had emerged and the parasites become more dispersed within the host population as they mature.

A. foliaceus is also called the 'carp louse' and it is not surprising therefore that the highest prevalence of lice was observed on carp. As with other host species, adult louse prevalence was higher than that of larval and juvenile life stages and this, combined with a relatively low average intensity, shows adult lice to be rather over-dispersed amongst the host population. Interestingly larval lice exhibited a very low prevalence and intensity. Carp are known to be fairly ubiquitous in their distribution within this lake and are probably relatively

more active than sticklebacks and gudgeon. This higher level of activity would make it more difficult for larval lice to locate and successfully attach to carp.

The differences between species were significant for several infection variables. The prevalence of the three life stages differed significantly between species in all cases. Significant differences were also found for mean infection intensities (although the differences were only marginally significant for larvae), suggesting average infection intensities are dependent upon host species. Intensity distributions also support the hypothesis, formulated from the prevalence and average infection intensity data, that *A. foliaceus* exhibits a stage-specific distribution within the host community. Mean intensities of all lice, and the larval and juvenile stages, on sticklebacks were significantly higher than on all other host species. This indicates that this species exhibits habits that increase its exposure to young *A. foliaceus* (Walker *et al*., 2007).

The physical and immune response characteristics of the host fish probably play a role in determining infection characteristics. Ecological and behavioural characteristics of the host species may create different opportunities for different life history stages of *A. foliaceus* to infest them and this is the more likely driving force behind the infection characteristics observed in natural systems. Mikheev *et al*., (2003) similarly suggested that it is the interplay between host and parasite behaviour that determines host-searching success of the parasite and subsequent observed parasite infection patterns. The range of species found harbouring *A. foliaceus* supports the view that this is a generalist parasite species and patterns of parasite distribution within mixed host species communities are likely to be determined by host-parasite encounter frequencies. Whilst the mechanisms employed for host location (e.g. olfaction, vision, mechanoperception) have been investigated previously (Mikheev *et al*., 1998, 2000, 2003, 2004) information is still lacking on how these sensory mechanisms may influence host selection by this parasite and by each of its life history stages.

The observed differences in abundance between the life history stages is probably attributable to the fact that larval, juvenile and adult lice are known to spend different amounts of time free-swimming (Stammer, 1959)., Pasternak *et al*., 2000, Mikheev *et al*., 2003) Adult male lice must leave their hosts to locate mates, and females must do so in order to deposit eggs (Walker *et al*., 2004). Further studies examining these off-host periods for lice, in particular for the adult stage, are required to determine the degree to which these off-host periods influence louse distributions in the wild.

Differences in parasite prevalence and/or intensity on one host species at one time do not necessarily demonstrate a preference for this species (Lester, 1984), but may in fact reflect

differences in the level of exposure of this species to the parasite due to other factors such as the relative abundance or distribution of that host species. In conclusion, *A. foliaceus* distributions within a community differ between host species and parasite life history stages, and ecological/behavioural traits of both host and parasite play a significant role in determining these distributions.

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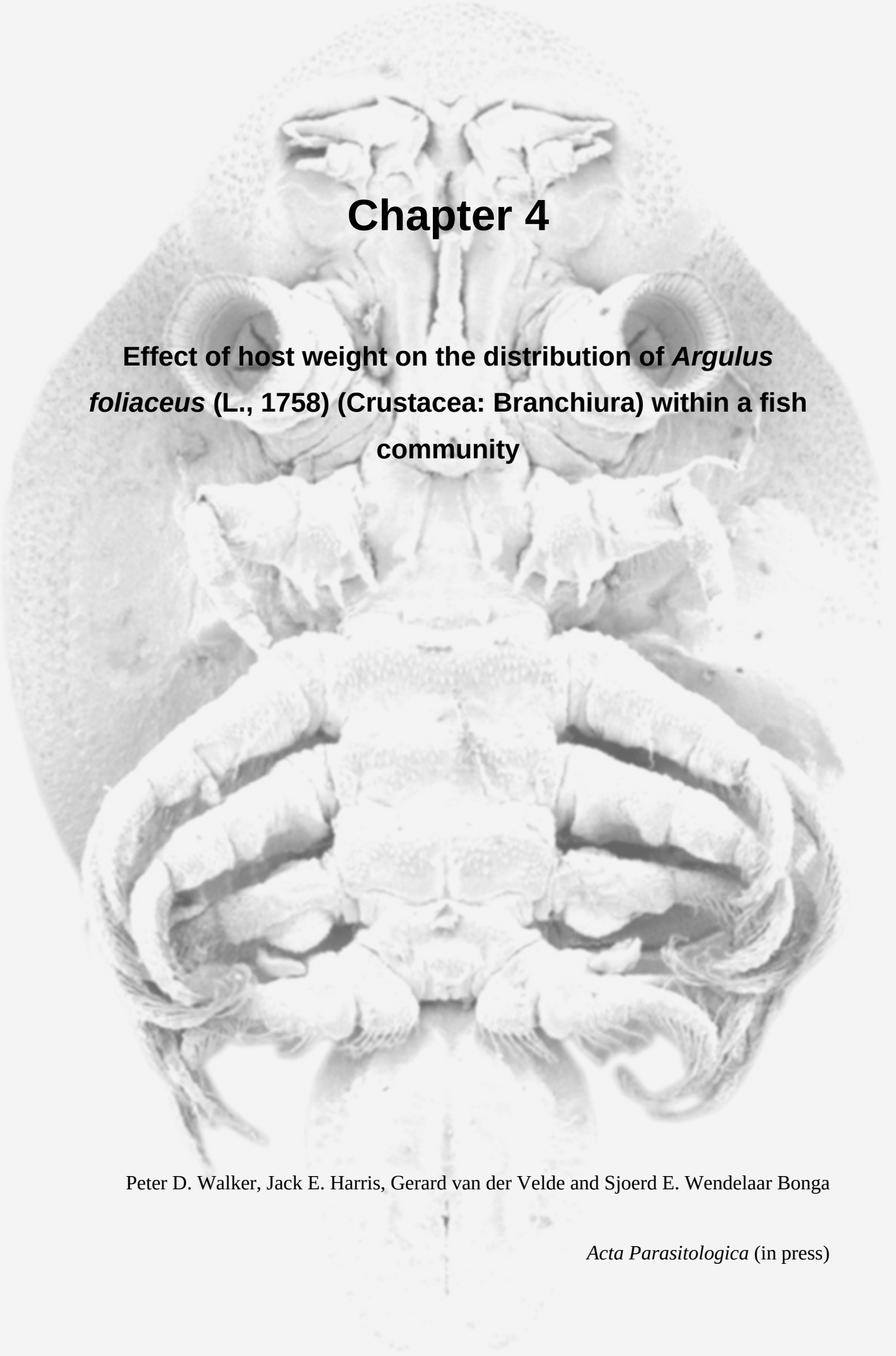

Chapter 4

Effect of host weight on the distribution of *Argulus foliaceus* (L., 1758) (Crustacea: Branchiura) within a fish community

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Abstract

Spatial heterogeneity is a feature common to many ecosystems. Aquatic organisms exhibit this heterogeneous distribution but to date little is known about the distribution of many common parasite species within water bodies. In this study the distribution of *Argulus foliaceus* (L.), an ectoparasitic crustacean, on different sized hosts within a mixed species fish community was determined.

Different fish species exhibited differences in their louse burdens (prevalence and intensity). The highest prevalence of *A. foliaceus* was observed on fish species dominated by larger individuals (i.e. *Cyprinus carpio*, *Abramis brama* and *Tinca tinca*). *C. carpio* and *A. brama* also exhibited the highest mean louse intensities.

Infested fish were generally heavier than uninfested conspecifics. Differences in the weight of uninfested and infested fish were significant ($P < 0.05$) for the whole fish community samples and the *Scardinius erythrophthalmus*, *A. brama* and *C. carpio* samples. There was also a general pattern of increasing infestation intensity with an increase in host body weight, with significant correlations for the whole fish community for *S. erythrophthalmus*, *A. brama* and *C. carpio* samples. In addition there were significant differences in parasite prevalence and intensity between different host weight groups and larger (heavier) fish appeared to be more frequently infested by, and harboured higher numbers of, *A. foliaceus*.

Introduction

Identifying the characteristics that result in observed abundance and distribution patterns of organisms is one of the central goals of ecology (Poulin, 1999b). Within infested host populations, parasite species often show a heterogeneous, highly-dispersed pattern with many individual hosts harbouring very few parasites and very few hosts harbouring large numbers (Poulin, 1993 *and references therein*). One of the main questions associated with this observation is: why do certain individuals exhibit higher levels of infestation than others? Factors determining observed infestation characteristics are typically complex and may be based on a variety of factors including morphological, physiological, behavioural, immunological, genetic or nutritional characteristics (Mustafa *et al.*, 2005).

Bandilla *et al*. (2005) suggested that for the fish louse *Argulus coregoni* Thorell (Crustacea: Branchiura) on rainbow trout, *Oncorhynchus mykiss* (Walbaum), infestation characteristics were determined, at least in part, by ecological and/or behavioural characteristics of the parasite as well as the host. They demonstrated that these characteristics led to an increased exposure risk to infective stages of the parasite for some individuals and suggested that differences in susceptibility linked, for example, to immune competence, were less important in determining observed infestation levels.

The size of individual hosts also influences parasite distribution within a host population. Grutter (1994) found a positive correlation between host fish length and gnathiid parasite loads. Rózsa (1997) showed that wing-feather mite abundance correlates with the body mass of their bird hosts. Poulin (1999b) demonstrated that for copepod ectoparasites of fish both parasite prevalence and intensity correlate positively with host body size. However, Poulin (2000) showed that the nature of the relationship between intensity of infection and host size depends on the individual host and parasite species involved.

From a parasites perspective, individual hosts are unequivocally islands (Kuris *et al.*, 1980). Using the MacArthur and Wilson (1967) island biogeography theory we would predict that larger hosts are likely to harbour more parasite species and higher numbers of individual parasites than smaller hosts (Kuris *et al*., 1980 *and references therein*). This is due to the probability that a larger host has the potential for a higher availability of resources. In addition, larger hosts are frequently older (Kuris *et al*., 1980) with a longer period of exposure to parasites. However, this parasite accumulation theory is probably not valid for parasites that frequently change their host, as is the case for intermittent parasites, or micropredators such as ectoparasitic fish lice.

LaMarre and Cochran (1992) suggest that parasites may be size-selective and, as a

result, host species preferences may be confounded with the effects of host size. Even if active selectivity is not apparent, then the fact that they attach to the hosts surface may mean that just by chance, larger hosts, providing a larger surface area, are attacked relatively more often than smaller hosts (Cochran, 1985). Adult fishes frequently exhibit different habitat preferences to their juvenile counterparts. LaMarre and Cochran (1992) state that because of these possible effects on host-selectivity, any study on host species preferences should control, or account for, host size.

Argulus foliaceus (L.) has been found on a wide range of freshwater fish species and is therefore frequently described in the literature as being a generalist parasite, non-selective in its choice of hosts (Kearn, 2004). However, this could simply be the result of host availability. In situations where equal numbers of equal sized hosts are present at the same time, host species preferences of *A. foliaceus* may become more apparent. Mikheev *et al.* (1998) studied this situation on roach, *Rutilus rutilus* (L.), and perch, *Perca fluviatilis* (L.), and found that perch were generally favoured over roach. There are only a few studies investigating host preferences by *A. foliaceus* and this could be due in part to its universal acceptance as a generalist. In this study on the distribution of *A. foliaceus* in a multi-species fish community, we address the question: are larger (heavier) fish more likely to be infested by *Argulus foliaceus* than their smaller counterparts?

Materials and methods

Fish sampling

Sampling took place in a lake of a mixed commercial coarse fishery in SW England (OS grid reference: SX456751) during the late spring and summer months of the year 2002, the seasons when *A. foliaceus* is most abundant (Walker *et al.*, 2004). Angling methods were used for sampling fish for the following reasons: i) the fishery owner wished it; ii) the authors felt this method of fish capture caused the least disturbance to the environment; iii) it did not cause excessive damage to the fins, scales, mucous layer and epidermis of the fish; iv) it reduced the risk of parasites being 'rubbed' off during capture; v) several regions of the fishery and several species of fish could be targeted simultaneously by using several anglers. During each sampling trip several different areas were fished and within each area anglers would fish at several depths and positions during the course of the day. Upon capture all fish were placed on a pre-wetted, white mat to aid spotting of dislodged parasites, and handled with wet hands to minimise damage to the epithelium and protective mucous layer. The eyes of larger fish

were covered with a damp cloth to help calm the fish during parasite collection.

Parasite collection

The external surfaces including buccal and gill cavities of all fish were examined thoroughly for *Argulus* individuals which were gently removed from their host using a set of blunt forceps. The total number of parasites collected from each fish was recorded along with the fish species and weight.

Data analysis

Differences between the average weight of infested and uninfested hosts were analysed with Student t-tests calculated in INSTAT. Where multiple pair-wise comparisons were conducted a Bonferroni-Holme correction was applied. A Spearman rank correlation test was used to examine the relationship between host weight and parasite intensity. This test is non-parametric and is more applicable when data do not fit a Gaussian distribution as was the case here. Spearman rank correlation tests were also calculated in INSTAT.

Parasite prevalence (= the proportion of the host sample that was infested) with better exact confidence limits and mean intensity (= the average number of lice per infested fish, excluding the zero values for uninfested fish) with bootstrap confidence limits as recommended by Rózsa *et al*. (2000), were calculated using Quantitative Parasitology 3.0 (QP 3.0: Reiczigel and Rózsa, 2005). Chi-square test (prevalence all fish) or Fishers exact test (prevalence individual species) were used to test for differences between the parasite prevalence of different groups and differences were deemed significant if the 2-sided P-value <0.05. Bootstrap 2-sample t-tests were used to test for differences between the mean infestation intensity of different groups. In each case 10,000 bootstrap replications were used and differences were deemed significant when the 2-sided bootstrap P-value <0.05. Bootstrap t-tests and Fisher's exact test were carried out using QP 3.0 (Reiczigel and Rózsa, 2005).

Results

Rudd *Scardinius erythrophthalmus* (L.), common bream *Abramis brama* (L.), tench *Tinca tinca* (L.), crucian carp *Carassius carassius* (L.) and common carp *Cyprinus carpio carpio* L. were caught during sampling. Table 1 shows the number of individuals of each fish species caught on each sampling date. The total number of individuals of each species caught and their mean weight are given in Table 2. Rudd (38.9 %) and common carp (35.6%) were far more numerous than the other species, accounting for approximately 75% of the total portion

of the samples.

Table 1. The number of individuals of each species caught on each sampling date.

Date	Rudd	Bream	Tench	Crucian carp	Common carp
08/05/2002	8	0	0	0	7
21/05/2002	17	6	0	1	10
05/06/2002	21	8	2	5	12
14/06/2002	12	6	1	0	13
25/06/2002	8	0	8	8	7
04/07/2002	29	8	0	7	24
10/07/2002	20	9	7	10	29
25/07/2002	20	0	0	0	27
07/08/2002	18	5	6	3	11

The fish size class distribution varied considerably within the community and between species (Fig. 1). The fish samples appeared to be dominated by fish of <200g although the very large fish (>500g) also made up a significant portion. The rudd population was dominated by very small fish (≤50g) with no fish >300g being caught. The bream population was dominated by fish in the 101-200g category which was similar for crucian carp. Bream ≤50g were not caught. There were relatively few small fish (≤100g) in the tench samples. The carp catches appeared to be dominated by two size groups, 101-200g and >500g. No carp ≤50g were caught.

All fish species harboured individuals infested with *A. foliaceus***.** Prevalence of *A. foliaceus* differed significantly ($P < 0.001$) between species (Table 2). Intensities differed significantly between bream and crucian carp ($P = 0.002$), carp and crucian carp ($P < 0.0001$) and carp and tench ($P < 0.0001$) (Table 2). After Bonferonni-Holme correction the differences between rudd and crucian carp ($P = 0.03$), and bream and tench ($P < 0.019$) were not significant although there was an obvious trend towards a higher infection intensity in rudd than in crucian carp and a higher infection intensity in bream than in tench.

The mean weight of infested fish was consistently higher than that of uninfested fish in all cases (Fig. 2). The differences between infested and uninfested groups were statistically significant ($P < 0.05$) for all fish together, and rudd, bream and carp, respectively, but not significant for tench and crucian carp ($P > 0.05$). Figures 3A-F show the relationship between

Table 2. Number of individuals for each host group, mean weight for each host group (± 1 s.d.), *Argulus foliaceus* louse prevalence and mean intensity (95% confidence intervals are given in parentheses).

Host Group	N	Mean weight (g)	Louse prevalence (%)	Mean louse intensity
All fish	393	266.2 ± 347.3	35.6	2.41
			(31-41)	(2.15-2.71)
Rudd	153	58.8 ± 52.9	8.5	2
			(5-14)	(1.46-2.54)
Bream	42	239.6 ± 176.2	40.5	2.47
			(26-56)	(1.82-3.06)
Crucian carp	34	212.2 ± 101.2	23.5	1.13
			(11-41)	(1.0-1.25)
Tench	24	435.0 ± 272.7	41.7	1.5
			(23-63)	(1.1-1.7)
Common carp	140	485.1 ± 460.2	65.7	2.66
			(58-73)	(2.3-3.11)

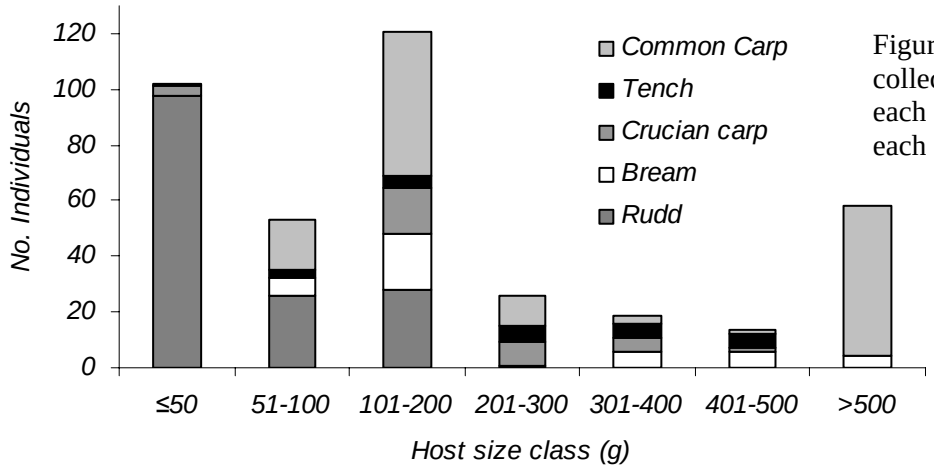


Figure 1. The number of collected individuals of each host species from each size group.

fish weight and infestation intensity on individual fish from each of the host groups. Significant correlations for host size and number of lice were found for all fish and for rudd, bream and carp sub-groups (Table 3). Correlation scores reflected a strong relationship between host size and number of lice for all fish and carp and bream sub-groups (Table 3). The relationship was less strong for rudd (Table 3). Correlations were not significant for crucian carp or tench as was reflected in their low r values (Table 3).

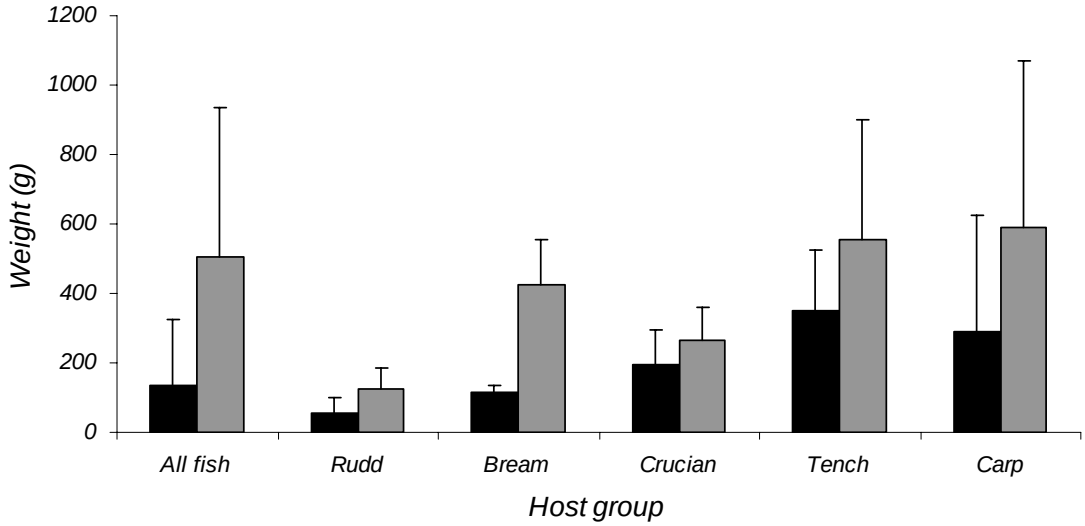
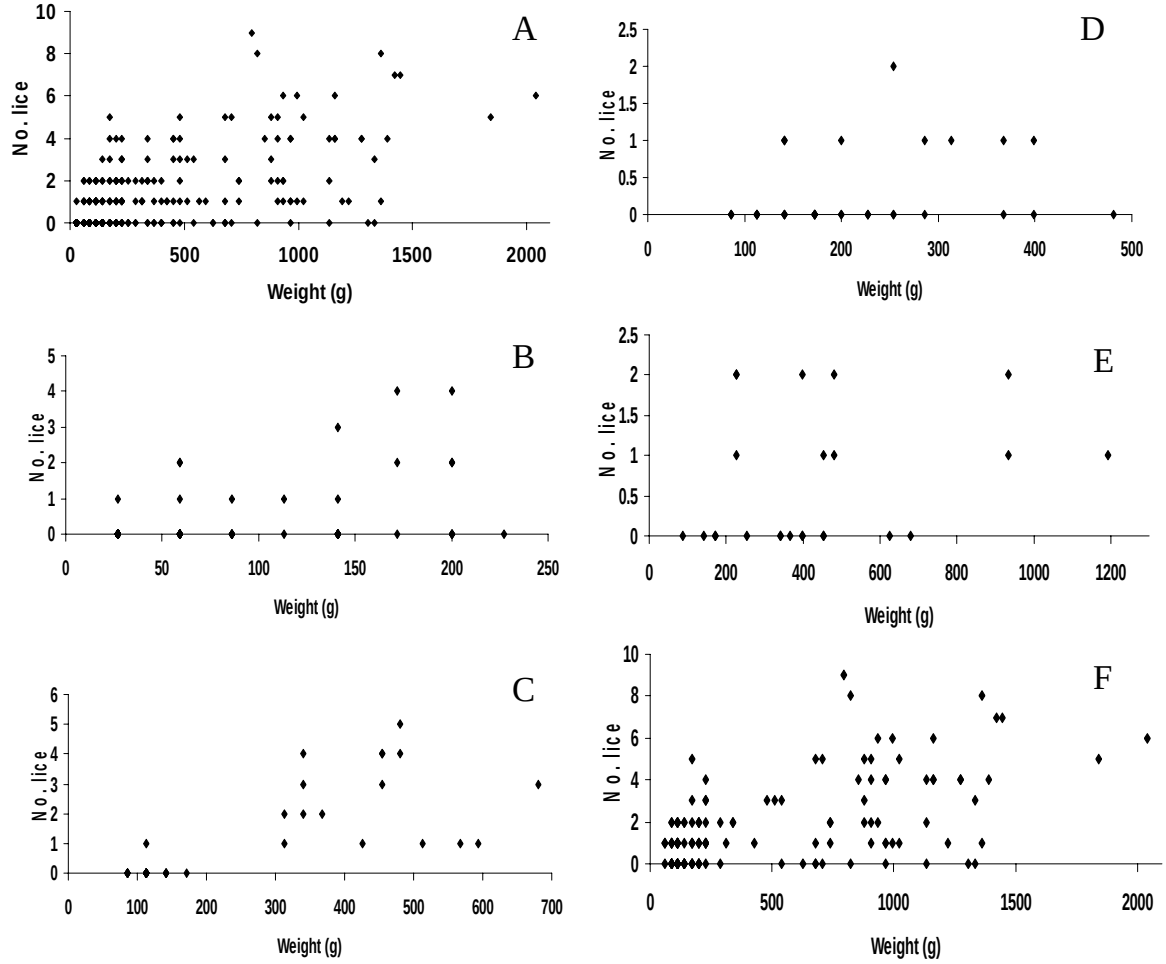


Figure 2. Mean weight in grams of uninfested fish (black bars) and fish infested by *Argulus foliaceus* (grey bars). Error bars = 1 s.d.



Figures 3A-F. The relationship between host weight and infection intensity by *Argulus foliaceus* for individual fish from the whole community (3A), rudd (3B), bream (3C), crucian carp (3D), tench (3E) and carp (3F) samples.

Table 3. Spearmans rank correlation coefficient scores, 95% confidence intervals and 2-tailed P-values for the relationship between host weight and infection intensity by *Argulus foliaceus*.

	All Fish	Rudd	Bream	Crucian carp	Tench	Common carp
r-value (corrected for ties)	0.4629	0.3908	0.8109	0.3113	0.2793	0.5271
95% confidence intervals	0.3172 - 0.5872	0.2430 - 0.5209	0.6676 - 0.8963	-0.04052 - 0.5944	-0.1524 – 0.6214	0.3916 – 0.6403
2-tailed P-value	<0.0001	<0.0001	<0.0001	>0.05	>0.05	<0.0001

Louse prevalence and intensity increased with increasing weight of the hosts (Figs. 4 and 5). Louse prevalence in the whole fish community differed significantly between host size classes (Chi-square statistic = 63.211, 5df, P <0.0001). Mean intensities however only differed significantly between 51-100g and >500g (P <0.0001), 101-200 and >500g (P <0.0001), 201-300g and >500g (P = 0.0003) and 301-400g and >500g groups (P = 0.0002).

All three size groups in the rudd samples were infested with lice (Table 4). Prevalence increased with increasing weight and the difference in parasite prevalence between groups was statistically significant (P <0.001). Mean intensity also increased with increasing host weight. Intensity data was only suitable for comparison for the 51-100g and 101-200g groups and here the differences were not statistically significant (P >0.05).

For bream differences between louse prevalence of the different size groups were statistically significant (P <0.001). Infestation intensity showed a trend towards higher intensities on larger fish although the intensity for the >500g size group was less than half that of the 401-500g size group. No significant differences were found between the infestation intensities of the different size groups although this could be due to small sample sizes.

For crucian carp no significant difference was found between the prevalence (P >0.05) or intensities (P >0.05 in all cases tested) of the different weight classes. Sample sizes for tench did not allow for meaningful statistical comparisons (Table 4).

All weight classes of carp were infested with lice (Table 4). Prevalence of lice on the different weight classes differed significantly (P <0.05). Infestation intensities showed a general trend of increasing with increasing host weight. Differences in infestation intensity between weight classes were however only significant between 51-100g and >500g (P <0.0001), 101-200g and >500g (P <0.0001), 201-300g and >500g (P = 0.002) and 301-400 and >500g (P = 0.002) groups.

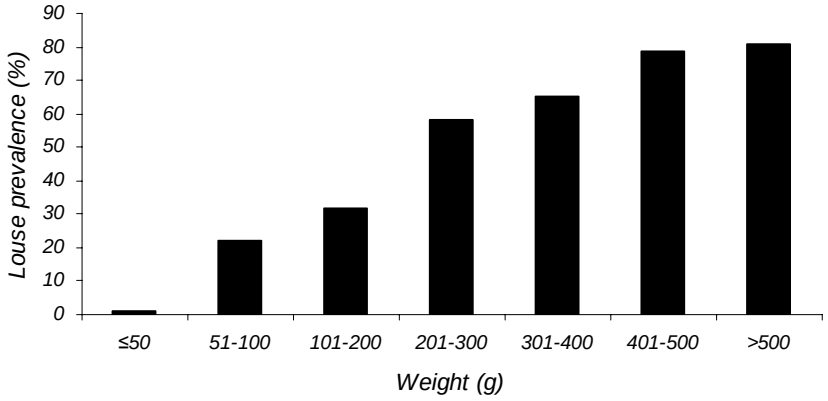


Figure 4. *Argulus foliaceus* louse prevalence on different size classes of hosts within the whole fish community.

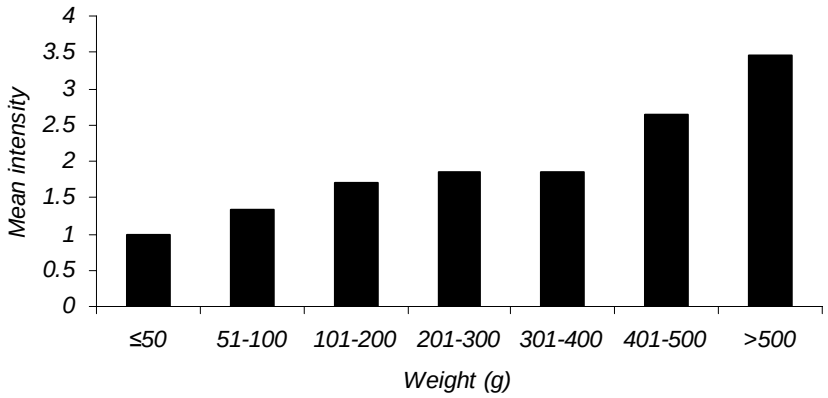


Figure 5. Mean *Argulus foliaceus* louse intensity on different host size classes within the whole fish community.

Parasite burden (number of lice/g) showed a trend of decreasing as host weight increased (Fig. 6). The data fit an exponential power curve. A Spearman rank (r_s) correlation test demonstrates a very significant (P <0.0001) negative correlation (r_s = -0.76, corrected for ties; 95% confidence intervals = -0.82 to -0.68).

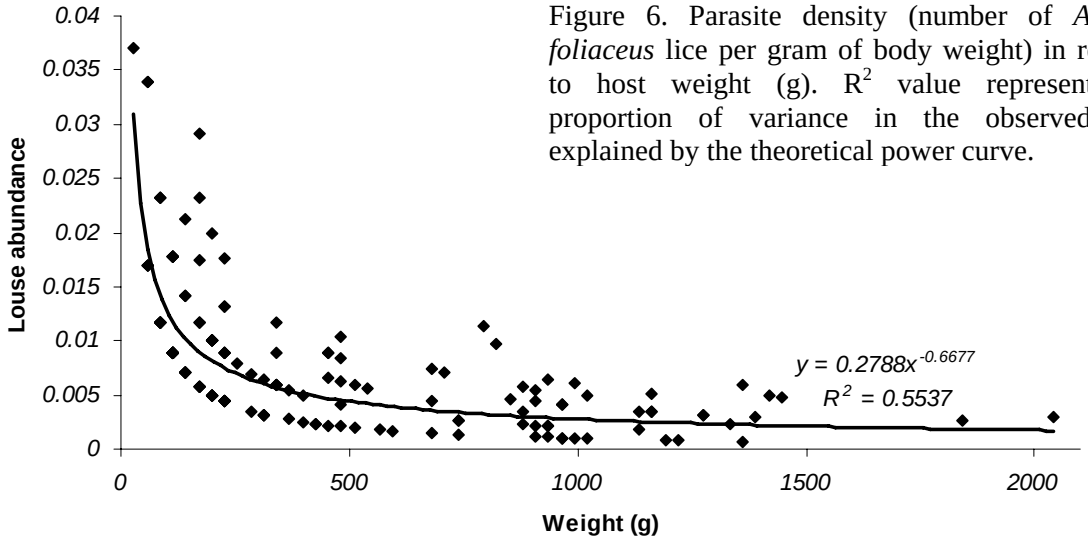


Figure 6. Parasite density (number of *Argulus foliaceus* lice per gram of body weight) in relation to host weight (g). R^2 value represents the proportion of variance in the observed data explained by the theoretical power curve.

Table 4. Summary infection statistics for rudd, bream, crucian carp, tench and carp sample groups.

Host species	Size category (weight g)	N	Prevalence (%)	95% confidence limits prevalence	Mean intensity	95% confidence limits intensity
Rudd	≤50	98	1.0	0.06 - 5.4	1.0	-
	51-100	26	15.4	5.4 – 34.4	1.5	1.00 – 1.75
	101-200	28	28.6	14.2 – 48.2	2.38	1.63 – 3.13
Bream	51-100	6	0	-	-	-
	101-200	20	5.0	0.26 – 24.4	1.0	-
	301-400	6	100	58.9 – 100	2.33	1.5 – 3.0
	401-500	6	100	58.9 – 100	3.5	1.83 – 4.17
	>500	4	100	47.3 – 100	1.5	1.0 – 2.0
Crucian carp	≤50	3	0	-	-	-
	101-200	17	17.6	4.99 – 41.7	1.0	-
	201-300	8	25	4.6 – 63.5	1.5	-
	301-400	6	60	18.9 – 92.4	1.0	-
	401-500	1	0	-	-	-
Tench	51-100	1	0	-	-	-
	101-200	3	0	-	-	-
	201-300	4	75	24.9 – 98.7	1.67	-
	301-400	6	16.7	0.86 – 58.86	2.0	-
	401-500	5	60	18.93 – 92.4	1.33	1.0 – 1.67
	>500	5	60	18.93 – 92.4	1.33	1.0 – 1.67
Carp	51-100	18	44.4	23.7 – 67	1.25	1.0 – 1.5
	101-200	52	50	36.5 – 63.6	1.62	1.35 – 2.04
	201-300	11	81.8	50.0 – 96.7	2.0	1.33 – 2.67
	301-400	3	100	36.9 – 100	1.67	-
	401-500	2	100	22.4 – 100	2.0	-
	>500	54	81.5	68.7 – 90.1	3.77	3.14 – 4.39

Discussion

The species encountered and size ranges were consistent with those that the fishery owner claimed to be stocked. Size ranges did not match those expected from a wild fish population (Bone *et al*., 1995) and this is due to the fact that this is a man-made, artificially stocked lake. Carp exhibited the highest mean weight. This species is deliberately favoured by coarse fisheries around the UK due to its ability to grow to large sizes relatively quickly.

The differences in louse prevalence and intensity between species suggest a preference of *A. foliaceus* for some host species. Carp appear to be the preferred host which is in agreement with Kollatsch (1959) and supports the use of the common name for *A. foliaceus*, the carp louse. Host availability may be a factor influencing the distribution of *A. foliaceus*. However, rudd was the most frequently encountered fish species from the lake (followed closely by carp) and this species exhibited the lowest prevalence of *A. foliaceus*. Additionally, tench were encountered the least often, but, exhibited the second highest prevalence of *A. foliaceus*. The high prevalence on tench is also in agreement with observations made by

Kollatsch (1959). This suggests that factors other than the number of available hosts within a water body are responsible for louse distributions within the fish community.

The higher mean weight of infested fish compared to uninfested fish indicates an increased risk of infestation with *A. foliaceus* for larger hosts. Differences were not significant for tench or crucian carp but it should be noted that these species were found in relatively low numbers (Table 1) and it may be that samples were biased due to this. This is supported by the relationship between host weight and infestation intensity as illustrated by the scatter plots (Fig 3A-F).

Prevalence of lice on different size classes generally showed significant differences, typically with larger host size classes exhibiting a higher prevalence. The low number of individuals from each size class for crucian carp and tench makes these analyses less reliable. However, for the other three species and the host community as a whole the pattern is relatively consistent, with larger fish typically exhibiting a higher prevalence and infestation intensity of *A. foliaceus*.

Throughout the literature various measures of host size are reported, e.g. length, weight and surface area. When estimating infection intensities, number of lice per unit of surface area is the most desirable method. However, accurately measuring this parameter for large numbers of fish under field conditions is usually not practical and therefore other measures of size are frequently used instead. We chose weight as this parameter correlates with surface area to a higher degree than standard length for fish according to Tucker *et al*. (2002) and O'Shea *et al*. (2006).

Ectoparasitic infestations generally show an increase in infestation intensity with an increase in host size (Tucker *et al*., 2002). This is probably related to the greater surface area available for attachment (Dogiel *et al.,* 1958). However, Todd *et al*. (2000) reported that there is no significant relationship between host size and infestation levels for wild adult Atlantic salmon (*Salmo salar* L.) and the ectoparasitc copepod *Lepeophtheirus salmonis* Krøyer. Tucker *et al*. (2002) showed that under experimental conditions larger fish acquired more lice but the relative density (number of lice per cm^{-2}) of lice was higher on smaller fish. Poulin *et al.* (1991) also demonstrated that host size, rather than host behavioural traits, determined the intensity of ectoparasitic infestations of *Salminicola edwardsii* (Olsson, 1869) on *Salvelinus fontinalis* (Mitchill, 1814). We also found that relative lice density (number of lice per gram body weight) was higher on smaller fish than on their larger counterparts. The debate in the literature over whether to use length or weight to estimate parasite burdens remains unresolved but the general consensus is that surface area is the best measure, at least for

external parasites such as lice, and weight correlates more closely with this variable than length in the majority of cases (O'Shea *et al*., 2006).

Poulin and FitzGerald (1987) concluded that *A. funduli* Krøyer (incorrectly identified as *A. canadensis* Wilson; see Poulin, 1999a) were not size-selective in their attachment to sticklebacks. However, *Gasterosteus wheatlandi* Putnam infested with the copepod *Thersitina gasterostei* Pagenstecher were significantly longer and heavier than uninfested individuals. LaMarre and Cochran (1992) suggest that the fact that *A. japonicus* Thiele readily attached to very small fish (34-62mm long) only days after having been attached to carp of 1-2 kg suggests that neither host size nor host species is of overriding importance in host selection.

Our data provide evidence that in general *A. foliaceus* show a higher prevalence and intensity on larger fish. To our knowledge this is the first paper to report on host size preferences by this species of *Argulus*. Poulin and FitzGerald (1987) suggest that it may be adaptive for *A. funduli* to parasitize smaller hosts because larger hosts capture and eat more free swimming parasites and the lice require more time and energy to penetrate the thicker skin layer of larger hosts. Mikheev *et al*. (2000) came to the conclusion that juvenile roach and perch usually avoid free swimming *A. foliaceus*. It is not known if adult fish exhibit the same behaviour. In addition, smaller fish generally occur in larger schools which may result in a dilution effect as shown by Poulin and FitzGerald (1989).

We propose that a combination of factors including physical characteristics (e.g. increased surface area for attachment and for visual location by parasites) and behavioural traits related to parasite detection and avoidance are responsible for observed parasite distributions amongst their hosts rather than a process of active size selection by *A. foliaceus*. However, our study does not enable us to conclude whether *A. foliaceus* is actively size-selective or not.

From this investigation we conclude that larger fish are more likely to be infested with *A. foliaceus*. We hypothesise that this is due to the fact that larger fish are easier for *A. foliaceus* to locate and attach to, due to their greater surface area. However, parasite burdens generally seem to be higher on smaller fish. Therefore, whilst larger fish may demonstrate an increased risk of infestation due to their size, the consequences of infestation may be more significant for smaller fish. In addition, we propose that behavioural differences related to parasite detection and avoidance may also be responsible, at least in part, for the observed parasite distributions.

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The authors wish to thank the members of the EU Training network, PARITY, for advice and comments during informal presentations of these data. Thanks are also extended to Ben Holbrook, Anthony Oxley, Iain Russon and Stewart Walker for assistance with fish sampling and parasite counting. In addition the authors wish to thank the owners and anglers at Mile Mead fishery for their assistance and tolerance. This is CWE publication 484.

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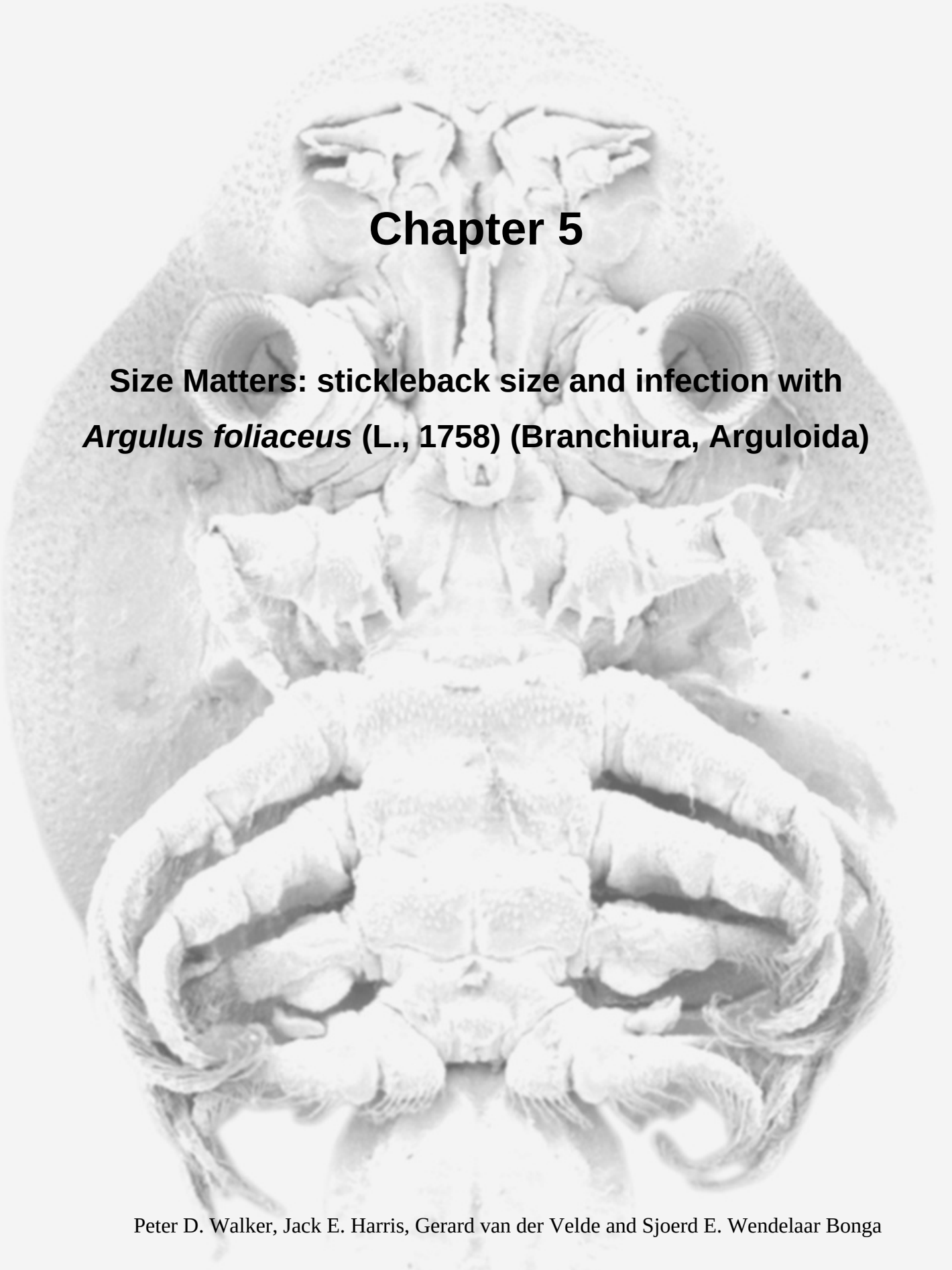

Chapter 5

Size Matters: stickleback size and infection with *Argulus foliaceus* (L., 1758) (Branchiura, Arguloida)

Peter D. Walker, Jack E. Harris, Gerard van der Velde and Sjoerd E. Wendelaar Bonga

Crustaceana 80, 1397-1405 (2007)

Short Communication

Parasite loads on fish have been shown to be influenced by the size of the host (Kabata, 1981; Grutter, 1994). The general trend is that a larger host will harbour higher numbers of ectoparasites (Tucker *et al*., 2002). The reasons for this may be partly related to the hosts age (larger hosts are generally older) with older hosts having had longer to accumulate parasites, and partly related to the surface area of the host as a larger host has a larger surface area making it easier for parasites to locate and attach to them (Kuris *et al*., 1980 *and references therein*).

Crustacean ectoparasites on fish provide an excellent model for studying parasite distributions within a host population due to the relative ease in identifying and counting them. Parasites from the genus *Argulus* are regarded as the most widespread and problematic parasites in freshwater fish culture (Kearn, 2004; Walker *et al*., 2004). This species has been responsible for significant economic losses in aquaculture (Menezes *et al*., 1990) and recreational fishery operations (Taylor *et al*., 2006). Despite this there are still huge gaps in our understanding of the way in which these parasites interact with their hosts.

Argulus foliaceus L. is regarded as non-host-specific and has been recorded from practically every freshwater fish species within its natural range (Stammer, 1959; Kennedy, 1974). Despite the opportunistic nature of this parasite some hosts still appear to be more susceptible than others (Bandilla *et al*., 2005). However, the factors influencing the distribution of *A. foliaceus* within a host population however are still poorly studied. In this investigation we analysed a stickleback population from a mixed species recreational fishing lake in the south-west of England.

Three-spined sticklebacks *Gasterosteus aculeatus* are common in many aquatic habitats in temperate zones of the northern hemisphere (Wooton, 1976). Because of their role in the food web as both predators and prey they are consequently hosts in many parasite life cycles (Kalbe *et al*., 2002). As a result they may also serve as reservoirs for some parasite species, transmitting parasites to other fish residing in the same habitats. Sticklebacks breed in the warm shallow regions of ponds and lakes (Davies *et al*., 2004), which are the same regions preferred for egg deposition by *A. foliaceus*. This will expose sticklebacks to this parasite throughout the breeding season and as such one would expect high infection prevalences and intensities on this species. However, juvenile sticklebacks appear to show behavioural changes including an increase in shoaling behaviour in the presence of argulid

lice (Poulin 1999). These changes in behaviour probably reduce the risk of individuals being parasitised.

Three-spined sticklebacks were caught from the littoral regions of the lake using a standard pond net. Upon capture the standard length of each fish was recorded and the number of attached parasites noted. Statistical analyses were carried out using Quantitative Parasitology 3.0 (QP 3.0: Reiczigel and Rózsa, 2005) and INSTAT.

53 sticklebacks were caught varying in length from 13 to 44 mm. 30 of the fish were infected with *A. foliaceus* giving a parasite prevalence of 56% (95% confidence limits : 42 to 70%). The mean infection intensity was 7.33 (95% confidence limits: 5.43 to 9.57; n = 30) and the maximum number of lice recorded on an individual fish was 23. The average length of infected sticklebacks was significantly greater (Mann-Whitney U-test, 2-tailed P-value < 0.0001) than that of the uninfected sticklebacks (Fig. 1). In addition, all infected fish were found to be greater than 30mm in length (Fig. 2).

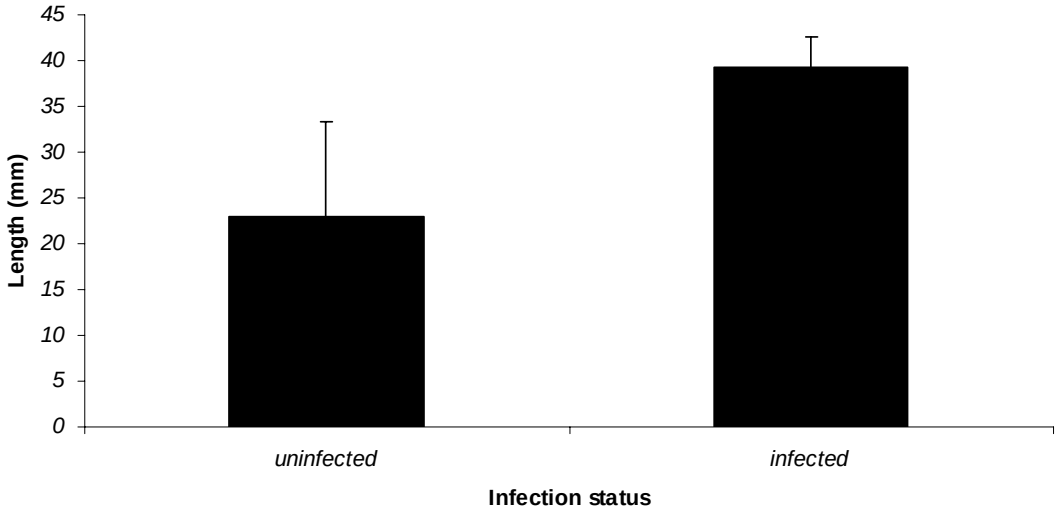

Figure 1. Mean standard length of infected (n = 30) and uninfected three-spined sticklebacks (n = 23). Error bars = ± 1 s.d.

Juvenile sticklebacks can and do exhibit parasite avoidance behaviour (Poulin and Fitzgerald, 1988; Dugatkin *et al.*, 1994). A similar behavioural trait was observed in juvenile roach *Rutilus rutilus* (Mikheev *et al.*, 2003). It is plausible that the lack of parasites on small sticklebacks in this study is a result of parasite avoidance behaviour. In addition it may be that *Argulus* infections are lethal to fish below a certain size and as a result any small fish that were infected perished so quickly that they were not accounted for in this study. *Argulus* can be lethal to juvenile carp *Cyprinus carpio* (Rhaman, 1996) and larval eels *Anguilla anguilla* (Hoffman, 1977). *Argulus* may also be size-selective in its choice of host, preferring to infect

larger individuals that are more likely to cope better with infestations than their smaller counterparts. It is also plausible that below a certain size threshold *Argulus* simply does not regard fish as a host. Poulin and Fitzgerald (1988) proposed that it may be adaptive for

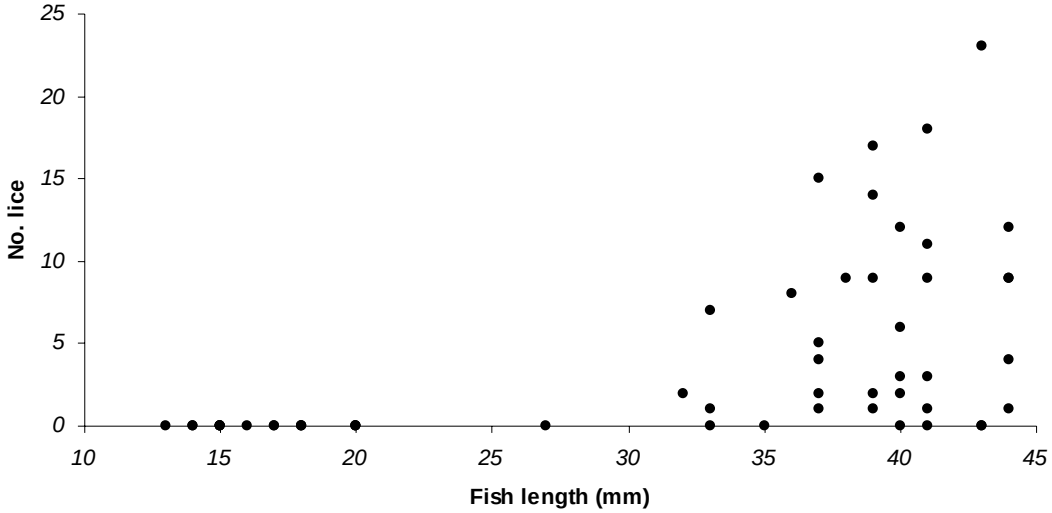

Figure 2. Scatter plot showing the relation between three-spined stickleback standard length and infection intensity.

Argulus canadensis to parasitise smaller hosts as larger fish capture and eat more parasites. However, our data for *A. foliaceus* and three-spined sticklebacks do not appear to support this hypothesis.

In conclusion, our study demonstrates a possible size-based difference in susceptibility amongst three-spined sticklebacks. Future studies involving the interactions between *A. foliaceus* and three-spined sticklebacks are required to elucidate the reasons for our observations.

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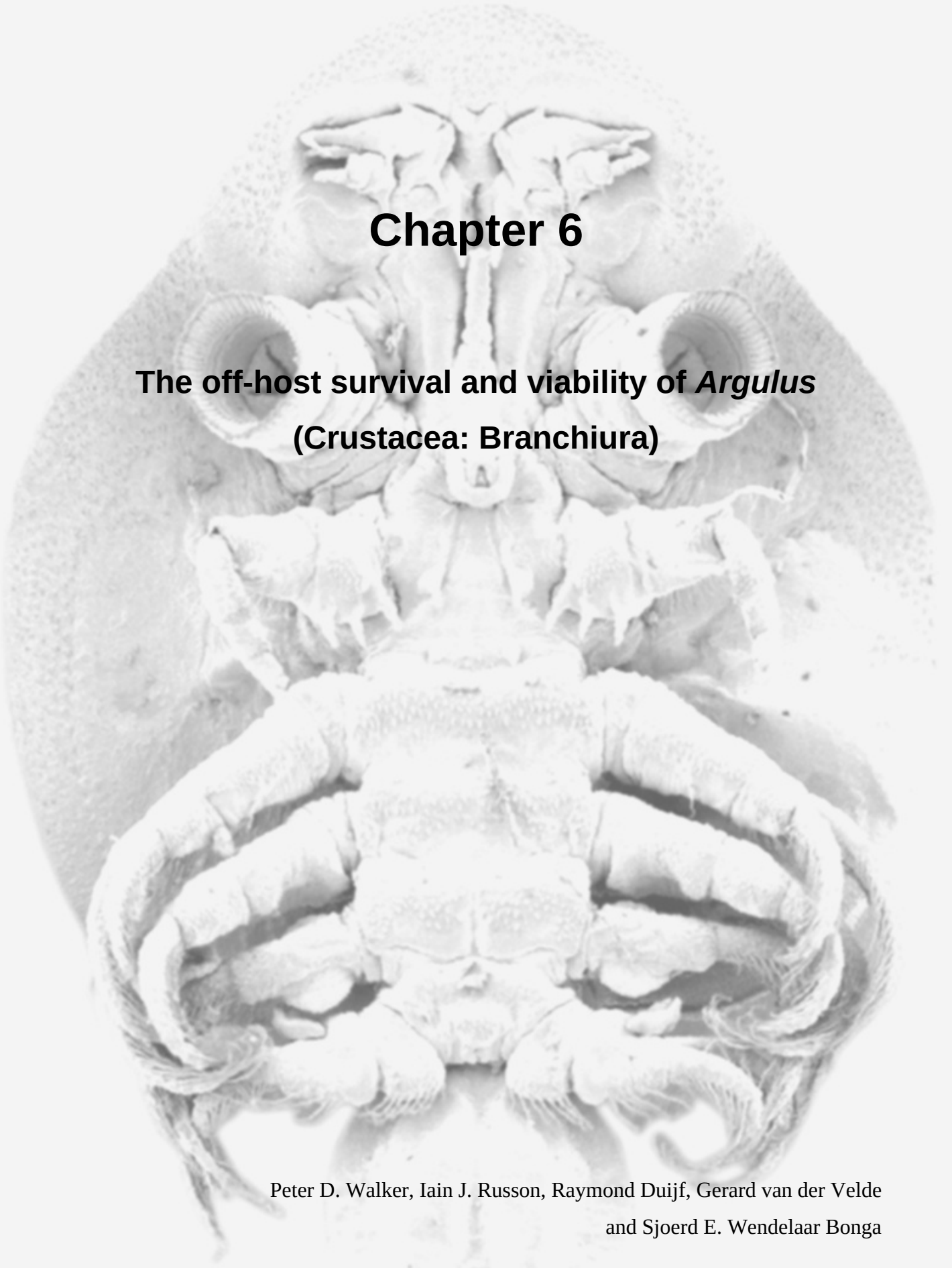

Chapter 6

The off-host survival and viability of *Argulus* (Crustacea: Branchiura)

Peter D. Walker, Iain J. Russon, Raymond Duijf, Gerard van der Velde and Sjoerd E. Wendelaar Bonga

Submitted to *Folia Parasitologica*

Abstract

During off-host periods, intermittent parasites must cope with varying abiotic conditions without access to food. We investigated the effect of temperature (5 to 28^{o}C) on the off-host survival time of *Argulus japonicus* Thiele, 1900, a crustacean ectoparasite of fish which is apparently expanding its distribution range, and compared this with its native European relative *A. foliaceus* (L., 1758). Results demonstrated a clear effect of temperature on the off-host survival time of larvae, juveniles and adults of both species. Larval and juvenile *A. japonicus* survived longest at 22^{o}C (up to 9 days) and adults at 15^{o}C (up to 13 days). Larval *A. foliaceus* survived longest at 15^{o}C (up to 5 days), adults at 9^{o}C (up to 14 days) and juveniles at 9 and 15^{o}C (up to 7 days). Thus, *A. japonicus* is more resistant to starvation at higher temperatures under off-host conditions and *A. foliaceus* is more resistant to starvation at lower temperatures. Infectivity of *A. japonicus* decreased linearly with time spent off-host after 2 days for larvae and 4 days for adults. Temperature only had a significant effect on the infectivity of both developmental stages after 24 hours off-host between 13 and 23^{o}C for larvae and 13 and 18^{o}C for adults. We conclude that temperature significantly affects off-host survival times and infectivity of argulids. Infectivity of *A. japonicus* is also influenced by time spent off-host.

Introduction

Parasites from the genus *Argulus* Müller, 1785, are the causative agents of the disease argulosis, which has been associated with fish spoilage and mortality of fishes world-wide. Damage typically occurs in the form of small craters formed by the feeding activities of the lice and epidermal hyperplasia at the wound margins (Walker *et al*., 2004). Wounds typically do not penetrate deeper than the epidermis although there are instances of wounds penetrating as deep as the stratum compactum (Lester and Roubal, 1995).

In addition to their destructive nature, *Argulus* species are known to act as vectors for other pathogens including viruses (Ahne, 1985), bacteria (Shimura, 1983), fungi (Bower-Shore, 1940, Stammer, 1959), and nematodes (Moravec, 1994). Bandilla *et al*., (2006) showed that argulids can also increase the susceptibility of their host to secondary infections. Reports detailing the damage and economic loss caused by argulids are widespread and involve several fish species which highlights the opportunistic nature of these parasites. *Argulus foliaceus* (L., 1758), is probably the most common and widespread argulid in Western Europe and to date is the most documented. This species has been reported as a threat to the culture of tilapia (*Oreochromis niloticus*) (Roberts and Sommerville, 1982, Paperna, 1991), rainbow trout (*Oncorhynchus mykiss*) (Menezes *et al*., 1990, Ruane *et al*., 1995) and common carp (*Cyprinus carpio*) (Jafri and Ahmed, 1994). Recently, Taylor *et al*., (2006) demonstrated that parasitic lice from the genus *Argulus* are also perceived as a potential economic threat to commercial recreational trout fisheries due to the damage they cause to fish and the resulting loss of revenue associated with a decrease in the number of anglers.

Argulus japonicus Thiele, 1900, is believed to have originated in Japan and probably owes its current wide-spread distribution to the trade in ornamental fish such as goldfish (*Carassius auratus*) and koi carp (*Cyprinus carpio*) (Rushton-Mellor, 1992). *A. japonicus* was not recorded from Britain until 1992, and was only known from a few isolated locations in the south. Nowadays, it is now probably widespread in the UK as well as in other parts of Western Europe (Rushton-Mellor, 1992). This species has also been reported from several locations in Africa despite the fact that it is also not endemic to that continent (Rushton-Mellor, 1994). This, and the lack of records of *A. foliaceus* from Africa, suggests a greater tolerance of *A. japonicus* for higher temperatures.

The life cycle of argulids was recently reviewed in Walker *et al*., (2004) and Kearn (2004). Larval lice hatch as free-swimming metanauplii from eggs that have been deposited on various substrates (e.g. plant stems and stones). Wilson (1902) observed yolk in the

digestive system of larval argulids but there is no data available on how long this yolk can sustain these lice. Reports vary with respect to the off-host survival times of newly hatched argulids but typically, state between 2 and 4 days (Mikheev *et al*., 2003).

Argulid fish lice when present on their host do not remain in contact with their hosts for the rest of their life. They may become dislodged by the fish or detach themselves for the purpose of mate location, egg deposition or to locate a more preferable host (Walker *et al*., 2004). The length of time spent apart from a host will ultimately depend upon the host location abilities of the parasites and the availability of suitable hosts. Reports regarding the survival time of adult lice during these off-host periods are fragmentary and sometimes conflicting. Factors such as timing of the parasites last meal, level of activity and temperature all probably determine this time period.

Despite the knowledge that species such as *A. foliaceus* and *A. japonicus* have a relatively ubiquitous occurrence and appear to be expanding their range (as is the case for *A. japonicus*) little is known about the effects of abiotic factors, including temperature, on these parasites, particularly with regard to their survival. This information could be vital in predicting the future spread of these potentially destructive lice, especially with the onset of global warming. In addition, in order to efficiently devise efficient, environmentally friendly control methods we need a thorough understanding of the biology of argulids and to identify environmental and biological factors which may naturally influence the success of such pathogens.

In this paper we investigate the effect of temperature and stage of development on the off-host survival of argulids by comparing two species, *A. japonicus* and *A. foliaceus*, with overlapping natural ranges, habitats and host species. In addition we discuss the effects of these off-host periods and temperature on the host location capabilities of *A. japonicus*.

Materials and methods

Parasite culture

Populations of *A. japonicus* and *A. foliaceus* were maintained on common carp *Cyprinus carpio* L. (approximately 1kg weight) in recirculation systems at 23^{o}C (± 1^{o}C) and with a 12:12 light:dark photoperiod. Nijmegen tap water (non-chlorinated) was used in all systems. Infestation intensities typically varied from 10 to 30 lice per fish. Eggs of *Argulus* were deposited on the glass sides and bottoms of the aquaria. Host fish were monitored regularly and parasite eggs were removed to control parasite numbers when infestation intensities

appeared to be too heavy as indicated by host condition and host-behavioural changes e.g. lethargy and loss of appetite. All parasite populations had been established under these conditions for several generations (exact number unknown) prior to these experiments.

Off-host survival of* A. japonicus *and* A. foliaceus: *effect of temperature

Adult *A. japonicus* and *A. foliaceus* (males >3mm, females >4mm to ensure maturity) were collected from stock carp that had been anaesthetised in a 2-phenoxyethanol (Sigma-Aldrich, St Louis, MO, USA) solution (dilution is 1:1000). Fish were considered anaesthetised once they displayed loss of equilibrium (i.e. turned 'belly-up') which took approximately 2 minutes. Parasites were subsequently removed from all fish using a set of blunt forceps and then held in beakers containing Nijmegen tap water (non-chlorinated) at 23^{o}C for 24 hours prior to use in experiments. During this time any eggs deposited by lice were collected and incubated in Nijmegen tap water at 23^{o}C with daily refreshment of the water. Upon hatching (the difference between hatching rates was less than 10 hours), larval lice (metanauplii) were either held for approximately 24 hours under the same conditions as adult lice or were allowed to attach to a group of juvenile common carp (maintained as for stock carp) to develop for 12 days before being collected and treated in the same way as for adult lice. At 23^{o}C *A. japonicus* and *A. foliaceus* take approximately 25 days to reach maturity (personal observations). Twelve day old lice are therefore considered as juveniles following the descriptions by Rushton-Mellor and Boxshall (1994). All lice used were taken from multiple egg strings and pooled prior to selection for subsequent experiments.

Lice from each of the developmental stages (larval, juvenile and adult; n = 30-50 lice per development stage, per trial depending on the number of lice available from cultures) were then placed into small clear plastic bags with 300 ml of Nijmegen tap water (23 ± 1^{o}C) and acclimated to experimental temperatures for 30 minutes before being added to experimental aquaria. Experiments were conducted in 3 replicate small glass aquaria (10 litres) containing Nijmegen tap water and maintained at 5, 9, 15, 23 or 28^{o}C (±1^{o}C) with a 12:12 light:dark photoperiod and without access to a host upon which they could feed. Aquaria were individually aerated and 10% of the water was refreshed daily after being acclimated to the appropriate temperatures. Parasites were examined daily and the number of dead specimens recorded. Death was assumed when no limb movements could be observed from the parasites, checked by gently touching lice with a small metal seeker and finally confirmed by examination under a binocular microscope.

Attachment success of* A. japonicus – *effect of starvation

Adult (24 days old) and larval lice (metanauplii – 1 day post-hatch) were acquired using methods described above. Fifty adult and fifty larval lice were held in beakers containing Nijmegen tap water (23 ± 1^{o}C) for varying periods of time (1, 2, 3, 4 or 5 days for larval lice and 1, 2, 3, 4, 5, 6 or 7 days for adult lice) without access to a host upon which they could feed. Parasites were then added to small (10 litre), individually aerated aquaria containing five juvenile common carp and left for one hour to locate and attach to the host. After one hour all carp were removed and anaesthetised and the number of attached lice recorded. The number of lice remaining in the aquaria (i.e. the ones that did not successfully attach to a host) was also noted to check if host predation on the parasites had occurred. Experiments were performed in triplicate (i.e. 3 x 50 lice for both larval and adult stages) for each period of time 'off-host'.

Attachment success of* A. japonicus – *effect of temperature

Adult (24 days old) and larval lice (metanauplii – 1 day post-hatch) were acquired using methods described above. Sixty juvenile common carp were equally distributed between 12 small (10 litre), individually aerated aquaria and acclimated to 13, 18, 23 or 29^{o}C (with 3 replications of each temperature), for a period of 2 weeks prior to use in experiments. Fifty lice were then placed into small transparent plastic bags containing 300 ml Nijmegen tap water and allowed to acclimate to experimental temperatures for a period of 30 min before being released into the aquaria to search for a host. All fish were sampled after 1 hour and thoroughly examined for any attached lice. The number of attached lice for each aquarium was then recorded and attachment success calculated as the % of lice successfully attaching to a host.

Data analysis

Differences between the off-host survival times of each of the developmental stages (larval, juvenile and adult) of the two parasite species (*A. japonicus* and *A. foliaceus*) were initially evaluated using 3-way ANOVA, with temperature, developmental stage and parasite species as factors. Data was then divided by species (*A. foliaceus* and *A. japonicus*) and evaluated using 2-way ANOVA with temperature and developmental stage as factors. Data was further subdivided by developmental stage (larval, juvenile and adult) and evaluated with 1-way ANOVAs. When these tests indicated significant differences in the data set Tukey's *post hoc* tests were used to determine where off-host survival times differed. Between species

comparisons were evaluated using 2-sample T-tests. Bonferroni-Holme corrections were applied to P-values where multiple pairwise comparisons were conducted within the same dataset.

Differences between the attachment success of larval and adult *A. japonicus* were evaluated with 1-way ANOVAs. When these tests indicated significant differences in the dataset Tukey's *post hoc* tests were used to determine where differences occurred. Differences in the attachment success of larval and adult *A. japonicus* at different temperatures were similarly evaluated using 1-way ANOVAs followed by Tukey's *post hoc* tests.

All data sets were tested for normality (Kolmogorov-Smirnov) and homogeneity of variances (Levene's) to ensure that data met the assumptions of statistical tests (ANOVA, Tukey's, T-test) prior to performing statistical tests.

Results

Off-host survival

The number of individual lice per treatment and developmental stage is shown in Table 1. Survivorship curves for *A. japonicus* and *A. foliaceus* larvae, juveniles and adults are shown in Figure 1. The majority of the curves exhibit a pattern somewhere between a type 1 and type 2 survivorship curve (Deevey, 1947). However, the data for juvenile and adult *A. japonicus* at 28^{o}C more closely resemble a type 3 survivorship curve (Deevey, 1947).

Considerable variation is exhibited between parasite developmental stages and temperatures. Maximum off-host survival times varied from 3 to 9 days for larval *A. japonicus* and 3 to 5 days for larval *A. foliaceus*. Juvenile lice showed a similar variation in maximum off-host survival times ranging from 5 to 12 days for *A. japonicus* and 5 to 7 days for *A. foliaceus*. Adult louse maximum off-host survival times ranged from 5 to 13 days for *A. japonicus* and 8 to 14 days for *A. foliaceus*.

The longest off-host survival times for larval lice were at 22^{o}C for *A. japonicus* and 15^{o}C for *A. foliaceus*. Juvenile lice survived off-host for the longest time at 22^{o}C for *A. japonicus*, and 9 and 15^{o}C for *A. foliaceus*. In the case of adult lice, *A. japonicus* survived off-host for the longest time at 15^{o}C and *A. foliaceus* at 9^{o}C. The mean off-host survival times of *A. japonicus* and *A. foliaceus* larvae, juveniles and adults at five different temperatures showed differences between developmental stages and at different temperatures (Fig. 2A and B).

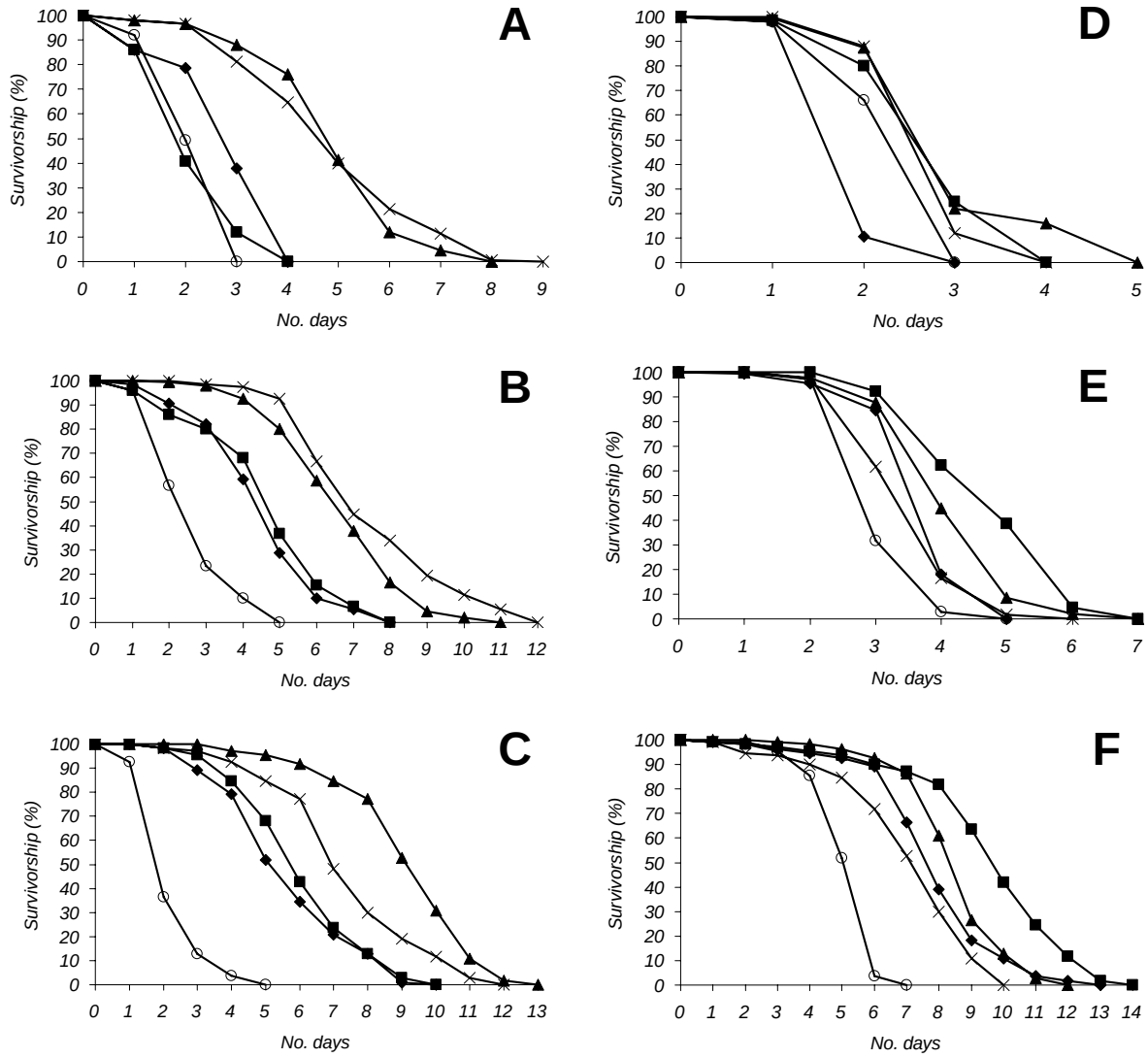

Fig. 1. Survivorship curves for three developmental stages of *A. japonicus* (larvae, A; juveniles, B; and adults, C) and *A. foliaceus* (larvae, D; juveniles, E; and adults, F) at five different temperatures. ♦ = 5°C; ■ = 9°C; ▲ = 15°C; × = 22°C; ○ = 28°C. Larvae = 1 day post-hatch (metanauplii); juveniles = 12 days old; adults = 24 days old.

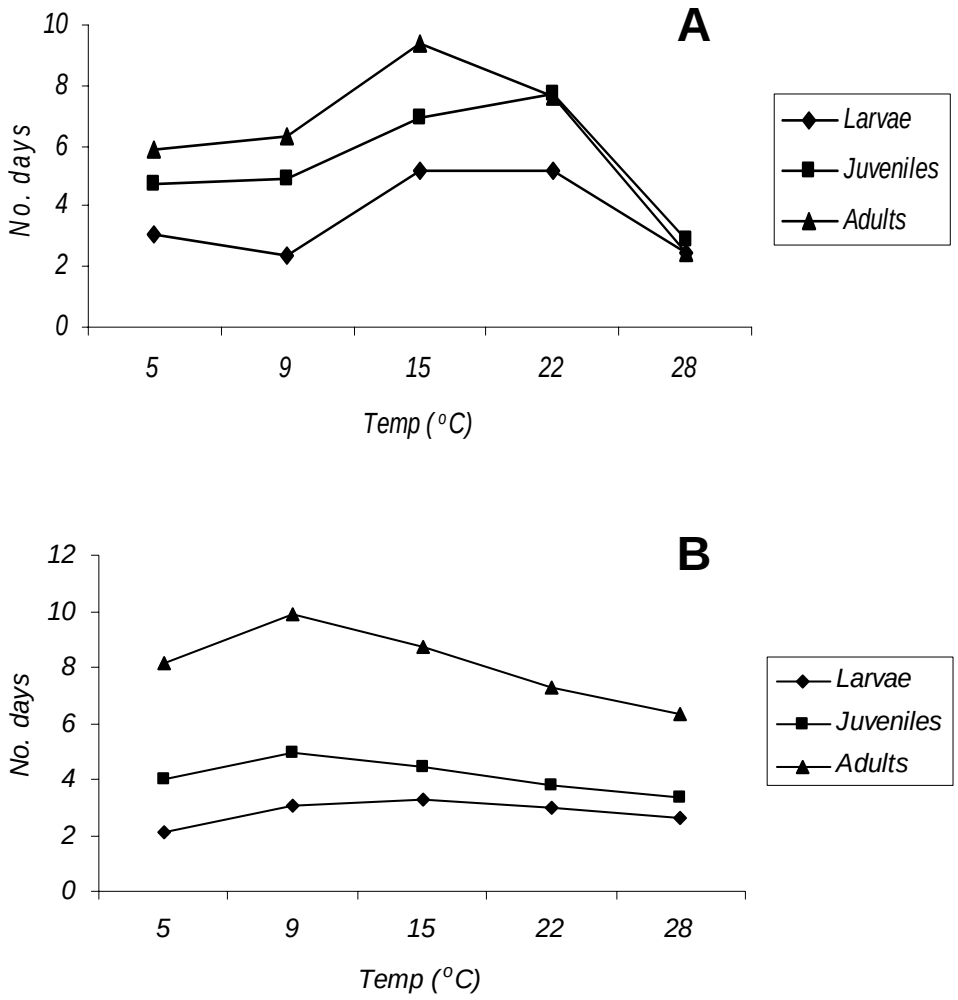

Fig. 2. Mean off-host survival time (in days), of **A** = *A. japonicus* and **B** = *A. foliaceus*, larvae (1 day-post-hatch), juveniles (12 days old) and adults (> 24 days old) at five different temperatures. Means were calculated from 3 replicate sets of up to 50 parasites. Standard deviations for *A. japonicus* ranged from 0 to 0.023 (larvae), 0.01 to 0.03 (juveniles) and 0.02 to 0.07 (adults). Standard deviations for *A. foliaceus* ranged from 0.01 to 0.04 (larvae), 0.03 to 0.09 (juveniles) and 0.05 to 0.1 (adults). Larvae = 1 day post-hatch (metanauplii); juveniles = 12 days old; adults = 24 days old.

Results of the 3-way ANOVA showed that the interaction variable Temperature x Developmental Stage x Species was significant ($F=216.719$, $P<0.001$, 8d.f.). Therefore data were divided by parasites species and further evaluated by 2-way ANOVAs. The interaction variable T x DS was significant for both the *A. japonicus* and *A. foliaceus* 2-way ANOVAs (*A. japonicus* $F=2763.23$, $P<0.001$, 8d.f.; *A. foliaceus* $F=305.35$, $P<0.001$, 8d.f.). Therefore data were subdivided by developmental stage and further analysed by 1-way ANOVAs.

Table 1. The number of *Argulus* lice per developmental stage used for trials examining the effect of temperature on off-host survival times (pooled from three replicates). Larvae = 1 day post-hatch (metanauplii); juveniles = 12 days post-hatch; adults = 24 days post-hatch.

Temp (^{o}C)		**5**	**9**	**15**	**22**	**28**
Larvae	*A. japonicus*	150	150	150	150	150
	A. foliaceus	150	150	150	150	150
Juveniles	*A.. japonicus*	150	150	150	150	150
	A. foliaceus	150	130	140	120	110
Adults	*A. japonicus*	110	110	110	110	110
	A. foliaceus	110	110	110	110	110

ANOVA showed significant differences between temperatures for the off-host survival times of larval *A. japonicus* ($F=22588.75$, $P<0.001$, 4d.f.). Tukey's pairwise comparisons revealed that these differences were significant between all temperatures except for between 9 and 28^{o}C and 15 and 22^{o}C (Table 2). ANOVA also showed that off-host survival times of juvenile *A. japonicus* were significantly different between temperatures ($F=20644.05$, $P<0.001$, 4d.f.). Tukey's test confirmed that differences in off-host survival times were significant between all temperatures for juvenile *A. japonicus* (Table 2). A similar result was obtained for adult lice with ANOVA showing significant differences between the off-host survival times of adult *A. foliaceus* at different temperatures ($F=10738.32$, $P<0.001$) and Tukey's test confirming that these differences were significant between all temperatures (Table 2).

For *A. foliaceus*, ANOVA showed significant differences between off-host survival times of larval lice at different temperatures ($F=1109.45$, $P<0.001$). Tukey's test showed these differences were significant in all cases for larval *A. foliaceus* apart from between 9 and 22^{o}C (Table 3). ANOVA also showed significant differences between off-host survival times of juvenile *A. foliaceus* at different temperatures ($F=329.21$, $P<0.001$) and between off-host survival times of adult *A. foliaceus* at different temperatures ($F=329.21$, $P<0.001$). Tukey's tests confirmed differences were significant between all temperatures for both juvenile and adult *A. foliaceus* (Table 3).

Table 2. Results of pairwise comparisons (Tukeys tests) to evaluated differences in off-host survival times between temperatures for the 3 developmental stages *A. japonicus*.

Temperatures	**Larvae**		**Juveniles**		**Adults**	
(^{o}C)	T	P-value	T	P-value	T	P-value
5 v 9	-48.00	<0.0001	7.4	0.0002	11.57	<0.0001
5 v 15	160.50	<0.0001	114.2	<0.0001	101.14	<0.0001
5 v 22	158.50	<0.0001	156.6	<0.0001	49.71	<0.0001
5 v 28	-46.00	<0.0001	-100.1	<0.0001	-97.41	<0.0001
9 v 15	208.50	<0.0001	106.8	<0.0001	89.6	<0.0001
9 v 22	206.50	<0.0001	149.2	<0.0001	38.1	<0.0001
9 v 28	2.00	0.3316	-107.5	<0.0001	-109.0	<0.0001
15 v 22	-2.00	0.3316	42.4	<0.0001	-51.4	<0.0001
15 v 28	-206.50	<0.0001	-214.3	<0.0001	-198.5	<0.0001
22 v 28	-204.50	<0.0001	-256.7	<0.0001	-147.1	<0.0001

Table 3. Results of pairwise comparisons (Tukeys tests) to evaluate differences in off-host survival times between temperatures for the 3 developmental stages *A. foliaceus*.

Temperatures	**Larvae**		**Juveniles**		**Adults**	
(^{o}C)	T	P-value	T	P-value	T	P-value
5 v 9	48.99	<0.0001	20.41	<0.0001	23.47	<0.0001
5 v 15	60.04	<0.0001	8.92	<0.0001	8.62	0.0001
5 v 22	47.27	<0.0001	-4.00	0.0167	-11.27	<0.0001
5 v 28	28.64	<0.0001	-13.25	<0.0001	-24.07	<0.0001
9 v 15	11.04	<0.0001	-11.49	<0.0001	-14.85	<0.0001
9 v 22	-1.73	0.4619	-24.41	<0.0001	-34.74	<0.0001
9 v 28	-20.36	<0.0001	-33.66	<0.0001	-47.54	<0.0001
15 v 22	-12.77	<0.0001	-12.92	<0.0001	-19.89	<0.0001
15 v 28	-31.40	<0.0001	-22.16	<0.0001	-32.69	<0.0001
22 v 28	-18.63	<0.0001	-9.245	<0.0001	-12.79	<0.0001

For both *A. japonicus* and *A. foliaceus* adult lice typically survive off-host for the longest periods and larvae for the shortest irrespective of temperature. Larval and juvenile *A. japonicus* generally survive longer off-host than their *A. foliaceus* counterparts. For adult stages however the trend is reversed with *A. foliaceus* surviving longer off-host at 5, 9 and 22^{o}C. *A. japonicus* adults survived longer than *A. foliaceus* adults at 15 and 22^{o}C however.

Off-host survival times were significantly different between *A. japonicus* and *A. foliaceus* for all developmental stages at all temperatures (Table 4).

Table 4. T-test results for analysis of differences in off-host survival times of the three developmental stages (larvae, juveniles and adults) of *A. japonicus* and *A. foliaceus* at 5 different temperatures. T-statistics (T), degrees of freedom (D.F.) and p-values are all shown.

		5°C	**9°C**	**15°C**	**22°C**	**28°C**
	T	34.2	-68.59	128.80	184.22	-17.0
Larvae	D.F.	3	4	2	4	3
	P	<0.001	<0.001	<0.001	<0.001	<0.001
	T	14.07	-5.63	62.81	211.40	-11.09
Juveniles	D.F.	2	2	3	3	2
	P	0.005	0.030	<0.001	<0.001	0.008
	T	-44.64	-111.64	13.01	4.04	-68.64
Adults	D.F.	2	2	2	3	3
	P	0.001	<0.001	0.006	0.027	<0.001

Attachment success of* A. japonicus *– effect of starvation

The two developmental stages differed in the relative numbers of lice successfully locating and attaching to a host within the one hour time frame of this experiment (Fig. 3). A maximum of 98% of all adult lice successfully located, and attached to, a host within one hour whereas for larvae the maximum value was substantially smaller at 74%. ANOVA showed significant differences in the attachment success rates of adult *A. japonicus* after different starvation periods ($F=2080.81$, $P<0.001$). Tukey's tests revealed that attachment success of adult *A. japonicus* started to significantly decline daily after approximately four days of starvation (Table 5). For larval *A. japonicus* ANOVA showed significant differences in the attachment success after different starvation periods ($F=946.50$, $P<0.001$). Tukey's tests show that attachment success of larval *A. japonicus* started to significantly decline daily after approximately 2 days of starvation (Table 5).

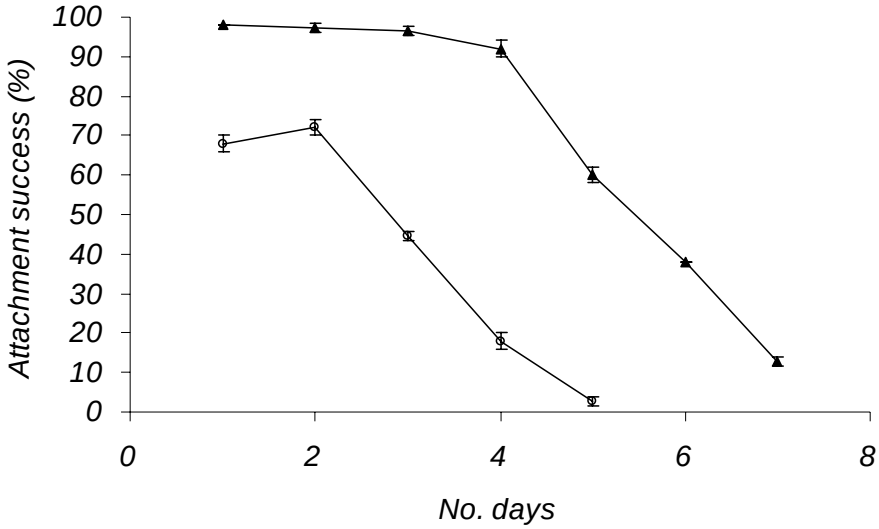

Fig. 3. Mean attachment success (% ± 1 S.D.) of larval *A. japonicus* (○) in relation to days post-hatch and the re-attachment success of adult *A. japonicus* (▲) in relation to days spent off-host. Larvae = 1 day post-hatch (metanauplii); juveniles = 12 days old; adults = 24 days old.

Attachment success of* A. japonicus *– effect of temperature

Attachment success was similar for both adult and larval lice and at different temperatures (Fig. 4). However, there were significant differences in the attachment success of larval *A. japonicus* at different temperatures ($F=16.53$, $P<0.001$). However, Tukey's test showed that this difference was only statistically significant between 13 and 23°C ($T=6.948$, $P=0.0003$). For adult *A. japonicus* ANOVA again showed that there were significant differences in attachment success at different temperatures ($F=16.53$, $P<0.001$). Tukey's tests, however, confirmed that the difference was only significant between 13 and 18°C ($F=5.964$, $P=0.008$).

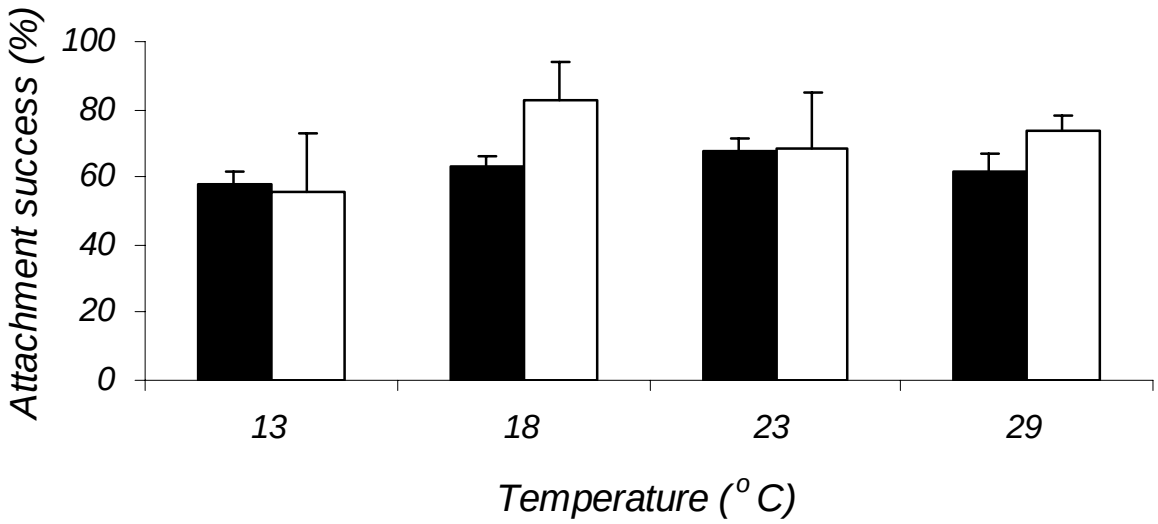

Fig. 4. Mean attachment success (% ± 1 S.D.) of *A. japonicus* adults (white bars) and larvae (black bars) at four different temperatures. Number of trials = 3 (50 parasites used in each trial therefore 150 adults and larvae/temperature). Larvae = 1 day post-hatch (metanauplii); juveniles = 12 days old; adults = 24 days old.

Table 5. Results of pairwise comparisons (Tukeys tests) to evaluate differences in attachment success of adult and larval *A. japonicus* after x days of starvation.

Adults			**Larvae**		
Starvation periods (days)	T	P-value	Starvation periods (days)	T	P-value
1 v 2	-0.62	0.9948	1 v 2	2.86	0.0971
1 v 3	-1.25	0.8641	1 v 3	-16.69	<0.0001
1 v 4	-5.61	0.0010	1 v 4	-35.75	<0.0001
1 v 5	-35.55	<0.0001	1 v 5	-46.72	<0.0001
1 v 6	-56.12	<0.0001	2 v 3	-19.55	<0.0001
1 v 7	-79.82	<0.0001	2 v 4	-38.62	<0.0001
2 v 3	-0.62	0.9948	2 v 5	-49.58	<0.0001
2 v 4	-4.99	0.0029	3 v 4	-19.07	<0.0001
2 v 5	-34.92	<0.0001	3 v 5	-30.03	<0.0001
2 v 6	-55.50	<0.0001	4 v 5	-10.96	<0.0001
2 v 7	-79.20	<0.0001			
3 v 4	-4.37	0.0089			
3 v 5	-34.30	<0.0001			
3 v 6	-54.88	<0.0001			
3 v 7	-78.57	<0.0001			
4 v 5	-29.93	<0.0001			
4 v 6	-50.51	<0.0001			
4 v 7	-74.21	<0.0001			
5 v 6	-20.58	<0.0001			
5 v 7	-44.28	<0.0001			
6 v 7	-23.70	<0.0001			

Dicussion

A major ecological difference between parasites and free-living animals is that once contact has been made with a suitable host, parasites no longer need to expend resources searching for food. As a result nutrient shortages are not a problem experienced by many parasites (Hakalahti *et al*., 2005). However, should a parasite lose contact with its host then nutrient shortages and the risk of starvation become significant issues in terms of parasite survival.

The off-host survival time of larval argulids were typically reported as approximately 2 days with maximum reported off-host survival times of approximately 4 days (Mikheev *et al*., 2003). Our data for *A. foliaceus* differ only marginally from those reported by Mikheev *et al*., (2003). The average off-host survival times determined by these authors ranged from 2.10 to 3.26 days with a maximum off-host survival time of 5 days (at 15^{o}C). Shafir and Oldewage (1992) stated that *A. japonicus* perished after only 3 or 4 days off-host. They did not, however, state the developmental stage of these lice. In our study, the mean off-host survival time of larval *A. japonicus* ranged from 2.39 days to 5.17 days (depending upon ambient temperature) which appears to be in rough agreement with the observations of Shafir and Oldewage (1992). The larval *A. japonicus* in our study survived for a maximum period of 8 or even 9 days off-host in a few cases. However, most of these animals appeared incapable of locomotion and could have been easily mistaken as dead, which may account for the differences between our data and those reported by Shafir and Oldewage (1992). Hakalahti *et al*., (2005) reported off-host survival times for larval *A. coregoni*, another European argulid, up to a maximum of 174 hours (approximately 7.5 days) with the majority of lice perishing after 90 hours (approximately 3.75 days) which is in closer agreement to our data for both *A. foliaceus* and *A. japonicus* than the data reported by Shafir and Oldewage (1992).

The differences observed between developmental stages and between temperatures for the off-host survival time of both *Argulus* species in our study may shed some light on the apparently conflicting reports found in the literature. Many of the reports regarding off-host survival times of argulids were based on observations under unspecified conditions with information regarding parasite developmental stage, time since parasite collection/hatching, light regimes and temperature not being reported.

We showed that the mean off-host survival time of adult *A. japonicus* ranged from 2.46 to 9.43 days with a maximum off-host survival time of 13 days (at 15^{o}C). Similar results were obtained for *A. foliaceus* although here mean survival times were higher than those of *A. japonicus* for 3 out of 5 of the temperatures and the maximum off-host survival time was 14 days (at 9^{o}C).

In general, adult *A. foliaceus* survived longer at lower temperatures than *A. japonicus*. Larval *A. japonicus* appear to be able to survive longer than larval *A. foliaceus* at temperatures in the mid-range of those studied here. The differences in the apparent optimum temperatures (in terms of off-host survival time) between the two species is interesting from an evolutionary perspective. *A. foliaceus* is native to Europe whereas *A. japonicus*, although widespread in Europe for many decades, is believed to have been introduced from the orient

via the trade in ornamental fish varieties (Rushton-Mellor, 1992). It may be that these two species have evolved in climates experiencing different temperature regimes and that as a result this has determined their temperature tolerance. With the continuing onset of global warming, this factor has implications for the future distribution of these two *Argulus* species.

Temperature has the greatest influence on off-host survival times at the two extremes of the parasites tolerance range but is most striking at the highest temperatures. *A. foliaceus* appears more adapted to survival at lower average temperatures typical of more northerly climates than its relative from the orient, *A. japonicus*. *A. japonicus* is reported as widespread across much of Southern and Western Europe (Walker *et al*., 2004). The latest British reports state its distribution in the United Kingdom is probably restricted to southern England currently (Rushton-Mellor, 1992) although by now it is likely this range has been extended. It can be hypothesized that the further spread of *A. japonicus* will be restricted by this species reduced ability to survive at lower temperatures although global warming may ultimately extend this species range. Whilst there are several reports of *A. foliaceus* overwintering as adults at temperatures lower than 5^{o}C, such reports are not available for *A. japonicus* or *A. foliaceus* larval and juvenile stages. Our experimental data demonstrate that all developmental stages of *A. foliaceus* and *A. japonicus* are probably also able to over-winter although their survival rates may be limited in comparison with adult *A. foliaceus* due to their shorter off-host survival time at lower temperatures. For both species high temperatures seem to be detrimental in terms of off-host survival times. This is again likely linked to the evolutionary history of these species as they both originate and persist in regions where such high water temperatures are not frequently experienced and thus they probably cannot tolerate prevailing conditions such as reduced dissolved oxygen levels typical of water at such temperatures. The effect of temperature on the metabolic process of these animals is not known but it is likely that extremes of temperature will also have a negative effect on such processes.

The short acclimation period used for lice in this study may have some influence on the results. In a natural situation temperature is unlikely to change as rapidly as experienced by lice used in this study. Acclimation of lice to different temperatures and the effects of acclimation periods on *Argulus* spp. off-host survival times is an area that ultimately requires further investigation.

We observed that 1 or 2 days before they perished, both argulid species became very inactive, sometimes even seeming to be dead until they were agitated with a metal seeker. Hakalahti *et al*., (2005) reported similar observations for larval *A. coregoni* (metanauplii) and attributed this to a possible state-dependent behavioural modification (Fenton and Rands,

2004). In our study, the attachment success of larval *A. japonicus* was optimal for approximately 2 days post-hatching and for approximately 4 days off-host for adults. The attachment success in relation to the time spent without access to a host upon which to feed, confirmed that lice become very lethargic after a certain period of starvation. In fact, several days before they die as a result of starvation, their ability to locate and infect a host is significantly diminished and at a certain point, although still alive, they do not appear to possess sufficient energy reserves to locate and attach to a host anymore and as a result can be considered not viable/infective any longer. We estimate this time frame to be after 4 days for larval *A. japonicus* and after 6 days for adult *A. japonicus* at 23^{o}C. We do not disagree with the state-dependent behavioural modification proposed by Hakalahti *et al*., (2005). However, at a certain starvation point behavioural modifications are probably over-shadowed by the lack of sufficient energy reserves to fuel any kind of host location behaviour. The difference in maximum numbers of lice successfully locating and attaching to a host fish hints towards a difference in the host location abilities of the different developmental stages. It appeared that adults are much more successful at locating a host than larvae. From this study it is difficult to conclude which mechanisms are responsible for this difference. We speculate that several factors likely play a role including the level of development of the sensory organs, swimming ability, morphological differences in attachment organs or perhaps even an ability of lice to 'learn' to recognise a host more quickly as they mature.

Kollatsch (1959) stated an optimal temperature for locomotion as being 18-23^{o}C for *A. foliaceus*. We expected to find that temperature therefore has a significant effect on host location and attachment success of *A. japonicus*. From our data it appears that the optimum temperature for host location and attachment success for *A. japonicus* is probably similar to that of *A. foliaceus* according to the results of Kollatsch (1959). From an evolutionary perspective it seems logical that a parasite would be minimally affected by temperature in terms of its ability to successfully locate and attach to a new host. Dislodgement or death of the host can occur during any season and therefore at any temperature, and any reduced ability to locate a host could be lethal to this parasite. Intermittent parasites need to retain their ability to mobilize themselves to locate and attach to a host under a range of environmental conditions and because >50% of parasites successfully located and attached to a host at each temperature, it appears that *A. japonicus* accomplishes this with respect to temperature.

Several authors have demonstrated that argulids modify their host-searching behaviour in order to best-utilize their energy reserves in relation to the likelihood of host encounters, i.e. they switch from sit-and-wait to active searching strategies connected with the changes

from light to dark conditions (reviewed in Mikheev *et al*., 2003). Hakalahti *et al*., (2005) propose a second type of host-searching behavioural modification that is state-dependent, i.e. dependent upon the state of the parasite's energy reserves. We observed that at a certain point, however, these mechanisms apparently fail and parasites lose the ability to successfully locate a host resulting in mortality.

In conclusion, we have shown that under experimental conditions temperature and developmental stage have a clear influence on the off-host survival time of *A. japonicus* as well as *A. foliaceus*. The off-host survival time increases as the lice mature. There were significant differences in the survival times of the two parasite species and each appears to have its own optimum temperature range, which also differs between developmental stages. We have also demonstrated that, like the native European *A. foliaceus*, *A. japonicus* can probably overwinter as adult stages increasing the potential number of generations produced in subsequent breeding seasons. In addition we have shown that off-host periods significantly influence the infectivity of *A. japonicus*, with lice becoming less viable the longer they have been without food. Finally, temperature does not appear to greatly influence the infectivity of *A. japonicus* within the broad temperature range tested although there may be a small increase in infectivity between the optimum temperatures of 18 and 23^{o}C.

We suggest that future studies regarding the survival abilities of parasites should always control or account for development stage and temperature. The information presented in this chapter has implications for the future management and control of *A. japonicus*. Control strategies must account for the fact that this parasite can probably survive as both egg stages and adult stages during the winter months and this poses a much larger threat in terms of the number of potentially infective lice appearing at the start of each breeding season if water temperatures in Western Europerise rise significantly as a result of global warming.

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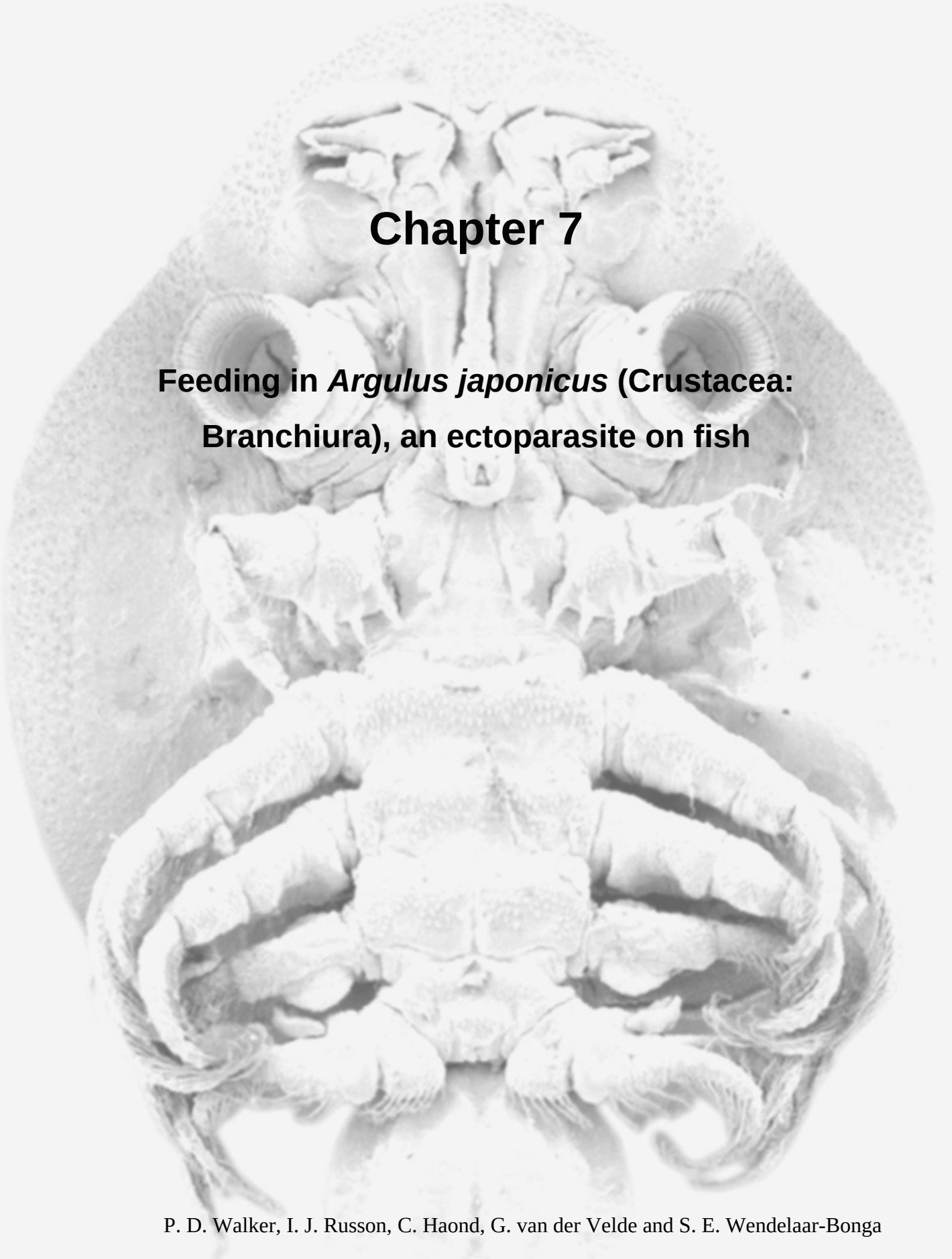


Chapter 7

Feeding in *Argulus japonicus* (Crustacea: Branchiura), an ectoparasite on fish

P. D. Walker, I. J. Russon, C. Haond, G. van der Velde and S. E. Wendelaar-Bonga

Submitted to Journal of Fish Diseases

Abstract

The argulids are an economically important group of crustacean ectoparasites known to be problematic in fish farming operations from both temperate and tropical regions. *Argulus japonicus* was first described parasitising Japanese fishes but is now well established in many parts of the world including Europe. To aid the development of effective chemotheraputants and/or vaccines against these parasites it is essential to know exactly what these animals ingest when feeding upon their fish hosts. From past morphological studies it has been shown that these parasites use a proboscis-like mouth tube for feeding, but, until now there has been much speculation regarding the diet of these animals. Live observations, histology, scanning and transmission electron microscopy were used to examine the feeding apparatus and gut contents of individual lice before and after feeding on juvenile common carp (*Cyprinus carpio*). Red colouration of the parasites gut after feeding was obvious suggesting ingestion of red blood cells by the lice. Sections of the lice used for histology and transmission electron microscopy revealed red blood cells from the fish host within the parasites gut. Haemorrhagic responses of the hosts skin was observed between 24 and 48 hrs post infection. All techniques provided evidence that these parasites are indeed obligate blood feeders.

Introduction

Ectoparasitc lice from the genus *Argulus* have negative impacts on the stock of fish farms and recreational fisheries (Menezes *et al*., 1990; Northcott, 1997; Taylor *et al*., 2006). Therefore these parasites have received considerable attention from researchers during the last few decades (Walker *et al*., 2004). In spite of this attention it is difficult to find clear statements regarding the feeding mechanisms and diet of these parasites (Kearn, 2004).

The feeding apparatus of *Argulus japonicus* and other argulids species have been relatively well described previously (Martin, 1932; Gresty *et al*., 1993; Kearn, 2004; Walker *et al*., 2004; Tam and Avenant-Oldewage, 2006). The mouth tube is composed of a labium and a labrum, which surround the buccal cavity. Contained within the buccal cavity are the mandibles and labial spines. In addition to the mouth tube there is a pre-oral stylet which has been the topic of some debate for several decades. To date the function of this unique organ is still unclear but the general consensus is that is used to deliver a toxin that may help breakdown the epithelial cells of the host or induce haemorrhaging. Similarly, the function of the labial spines is unconfirmed.

There is much controversy in the literature over the diet of *Argulus* spp. Authors state that *Argulus* spp. feed on fish mucous (LaMarre and Cochrane, 1992), skin (van der Salm *et al*., 2000) externally digested cell contents (Lester and Roubal, 1995) and tissue fluids (Frabioni, 1974). Others state that these animals are in fact obligate blood feeders (van Duijn, 1956; Ahne, 1985; Poulin and Fitzgerald 1988; Mikheev *et al*., 1998, 2000; Pasternak *et al*., 2000) but even here some authors have argued that they only ingest blood serum and not whole blood (Ivanfi, 1926: referenced in Gresty *et al*., 1993). This controversy is not altogether surprising because there has been a distinct lack of detailed studies to show exactly what these animals are feeding upon.

The aim of this study was to provide evidence on the diet of *A. japonicus*. Live observations, histology, scanning and transmission electron microscopy were used to examine the feeding apparatus and gut contents of *A. japonicus* after they had fed on juvenile common carp (*Cyprinus carpio*). Results are discussed in relation to the importance of parasitic feeding mechanisms and diet for the development of effective control methods.

Materials and methods

Feeding experiment

Adult *A. japonicus* were collected from Nijmegen laboratory brood stock carp using blunt forceps. These lice were then held in a small beaker containing aquarium water for 48 hours at 20^{o}C, without access to a host. The water was replaced once after 24 hours. Several lice were then taken and allowed to feed on a juvenile carp (*Cyprinus carpio*) for 2 hours before once again being removed with a set of blunt forceps. Several fish were left with lice attached and examined at 24 and 48 hrs post infection for gross pathological signs associated with parasitic infection. Live observations were then conducted by placing several fed and unfed parasites on microscope slides in a drop of water. The slide was placed in a freezer (-20^{o}C) for approximately 1 minute to reduce the level of activity exhibited by the animals. Low power micrographs were obtained using a stereo-microscope with a mounted digital camera.

Histology

Lice were collected after feeding on juvenile common carp for 2 hrs and immediately placed in Bouin's fixative for approximately 24 hrs. Samples were then processed for paraffin histology using the standard procedure. Paraffin sections were stained with Masson trichrome (Martoja and Martoja, 1967).

Skin samples were dissected from euthanised (overdose of 2-phenoxyethanol anaesthetic followed by spinal transaction) juvenile common carp (*Cyprinus carpio*) and immediately fixed in Bouin's fixative for a minimum of 24 hrs. Samples were subsequently dehydrated through a graded ethanol series and embedded in paraffin. Five μm sections were mounted on gelatinised glass slides and dried overnight at 40^{o}C. Slides were then stained with hematoxylin and eosine, and examined microscopically.

Scanning electron microscopy (SEM)

Adult and larval lice were collected from Nijmegen brood stock carp and then immediately fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and left overnight. Samples were then post-fixed for 2 hrs in ice-cold 1% OsO_4, 0.15 M sucrose in the same buffer before being dehydrated through a graded ethanol series, critical point-dried and sputter-coated with a microlayer of gold palladium. Specimens were observed with a JEOL SEM 6330.

Transmission electron microscopy (TEM)

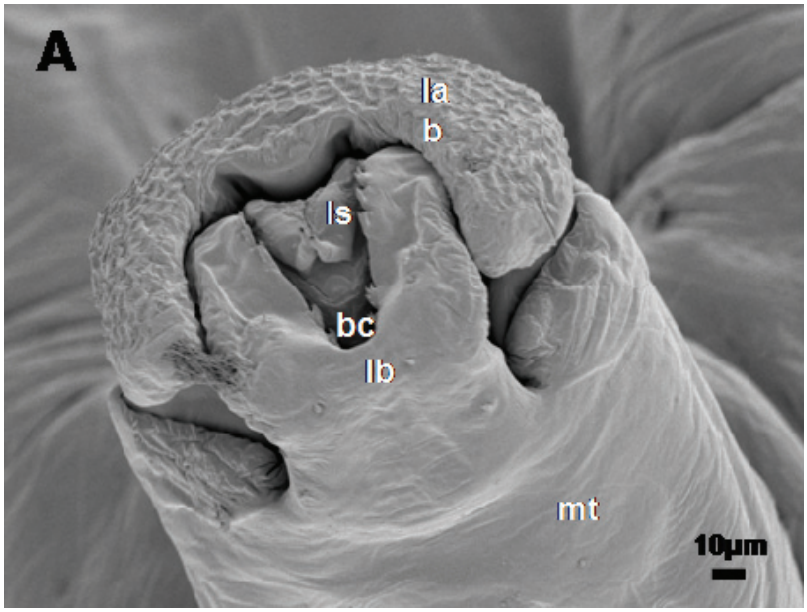
For electron microscopy, lice were collected after feeding for 2 hrs on juvenile common carp. The lice were then dissected in 2% glutaraldehyde in 0.1 M cacodylate buffer and kept for 4 hrs in ice-cold fixative. After washing in 0.2 M sucrose in 0.1 M cacodylate buffer, dissected pieces of the dorsal shield (carapace) were post-fixed for 2 hrs in ice-cold 1% OsO_4, 0.15 M sucrose in the same buffer, washed in demineralised water, dehydrated in ethanol baths and embedded in LR White resin. Ultra-thin sections were cut using a diamond knife (Diatom), contrasted with uranyl acetate and lead citrate, and examined with a JEOL CXII transmission electron microscope.

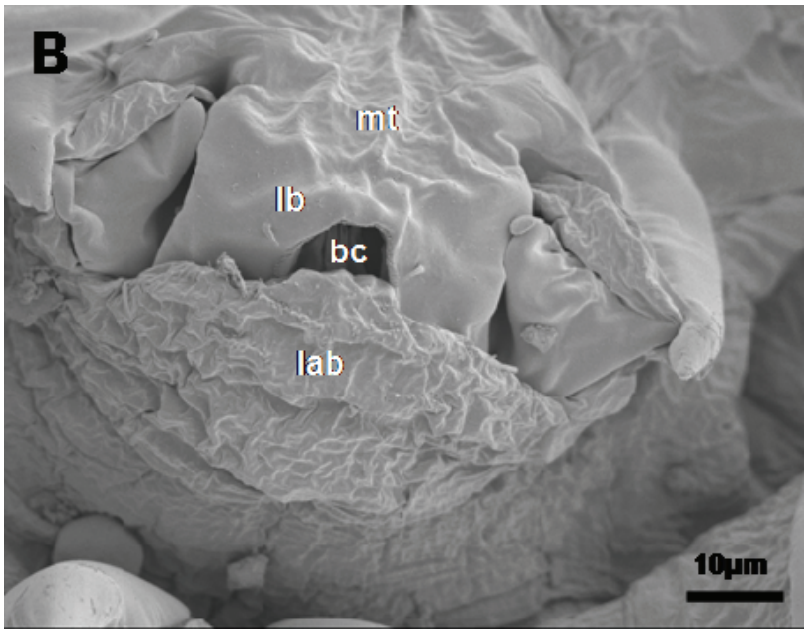
Results

Comparison of the feeding apparatus of larval and adult *A. japonicus* shows that the mouth parts are essentially comprised of the same component parts, e.g. mandibles, labrum and labium (Figs. 1A and B). The most notable difference between the feeding apparatus of adult and larval lice is in the overall size of the mouth parts. In particular the aperture of the buccal cavity is significantly larger in adult lice (approx 20μm across) than in larval lice (approximately 9μm across) (Figs. 1A and B). The pre-oral stylet consists of a spine possessing a rounded tip with two small openings located behind it (Fig. 1C). This stylet was either completely retracted inside its associated sheath or partially exposed (Fig. 1C), depending on the specimen.

The anterior and posterior midgut and enteral dirverticula are clearly visible in live *A. japonicus* larvae (Fig. 2F). The gut of larval *A. japonicus* have many pigment cells associated with it. Squash preparations of larval parasites after having been attached to juvenile carp for 2 hrs did not contain carp erythrocytes.

Examination of live adult parasites using the stereo microscope revealed that after 48 hrs without feeding the gut of these animals was completely empty (Fig. 2 A, B). The gut of these animals consists of a foregut or crop, and a hind gut (Fig. 2). There are also many diverticula running through the dorsal carapace which begin from one of two lateral extensions of the crop, one on each side of the animal. Numerous chromatophores associated with the gut diverticula were also apparent (Fig 2). Just two hours after being allowed to attach and feed on a small juvenile common carp, many of the parasites exhibited a conspicuous red colouration within the various regions of the gut suggesting that haematophagous activities had occurred and that the animal's gut was now full of blood.





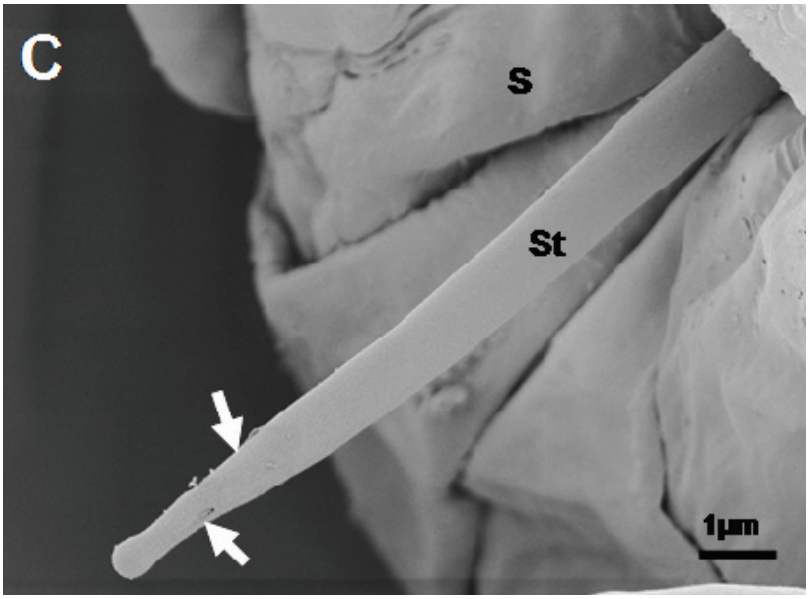


Fig. 1. Scanning electron micrographs of the mouth parts of an adult (A) and larval (B) *Argulus japonicus* showing the mouth tube (mt), labium (lab), labrum (lb), labial spine (ls) and buccal cavity (bc). The pre-oral stylet of an adult (C) takes the form of a hollow spine (St) possessing two openings near to its tip (white arrows) and is contained within a sheath (S) into which it can be retracted when not in use.

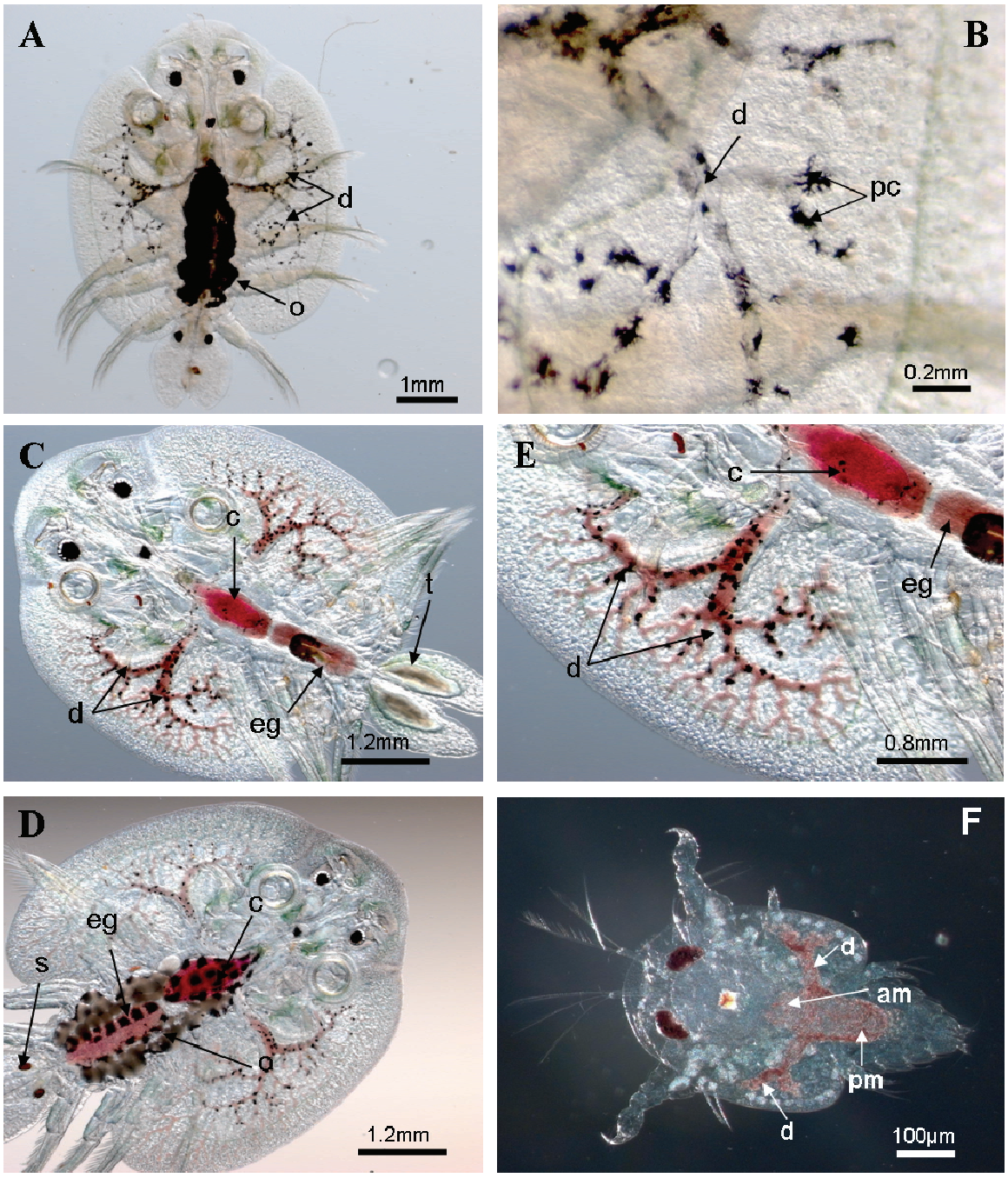


Fig. 2 A-F. Stereo micrographs of live *Argulus japonicus*; c, foregut or crop; eg, hind/end gut; o, ovaries; t, testis; s, spemathecae; d, enteral diverticula; pc, pigment cells (chromatophores); am, anterior midgut. (A, B) Female specimen observed 48hrs after removal from its host. The ovaries are clearly visible as are the diverticula of the gut extending throughout the dorsal carapace. The empty diverticula can be seen along with associated pigment cells or chromatophores (B). (C-E) Male and female specimens removed from their host after feeding for two hours. The male can be identified by the testis which are clearly visible within the abdominal lobes at the posterior end of the animal (C). The female specimen is distinguished by the conspicuous ovaries and also by the spermathecae which can be seen on the abdominal lobes (D). In both specimens blood can be seen in the foregut or crop, the diverticula of the gut and in the end gut. (E) Magnified area of the male parasites dorsal carapace showing in detail the diverticula filled with blood. (F) Larval louse 2hrs post hatch with the anterior and posterior portions of the midgut (am and pm respectively) clearly visible as are the enteral diverticula (d). Numerous pigment cells were associated with the gut of larval *A. japonicus* giving it a red/brown colouration here.

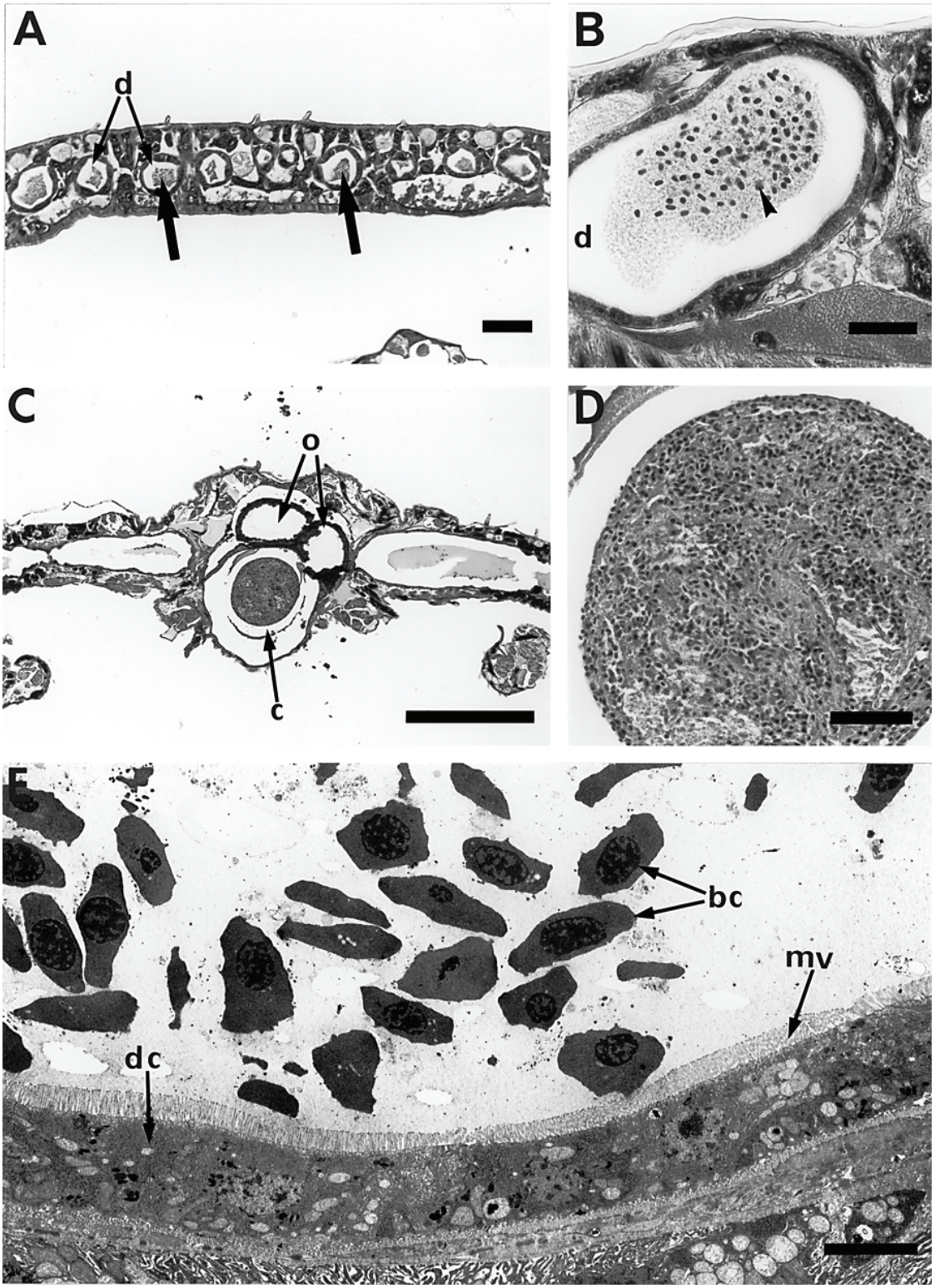


Fig. 3 A-E. Gut content of fed adult *Argulus japonicus*. (A) Longitudinal section through the carapace. Diverticula of the gut (d) seen in transverse section contain clotted material (arrows). (B) Transverse section through a gut diverticulum (d). Fish erythrocytes are clearly visible within the gut (arrowhead). (C, D) Transverse sections of the crop (c). Ovaries (o) can be seen clearly indicating the specimen was female. The crop contains a pellet (C) full of fish red blood cells (D). (E) TEM micrographs of a transverse section through the gut diverticulum. Digestive cells (dc) and microvilli (mv) surrounding the gut are clearly visible. Fish red blood cells (bc) are clearly identifiable within the gut. Scale bars: A-100µm, B-50µm, C-500µm, D-50µm, E-5µm.

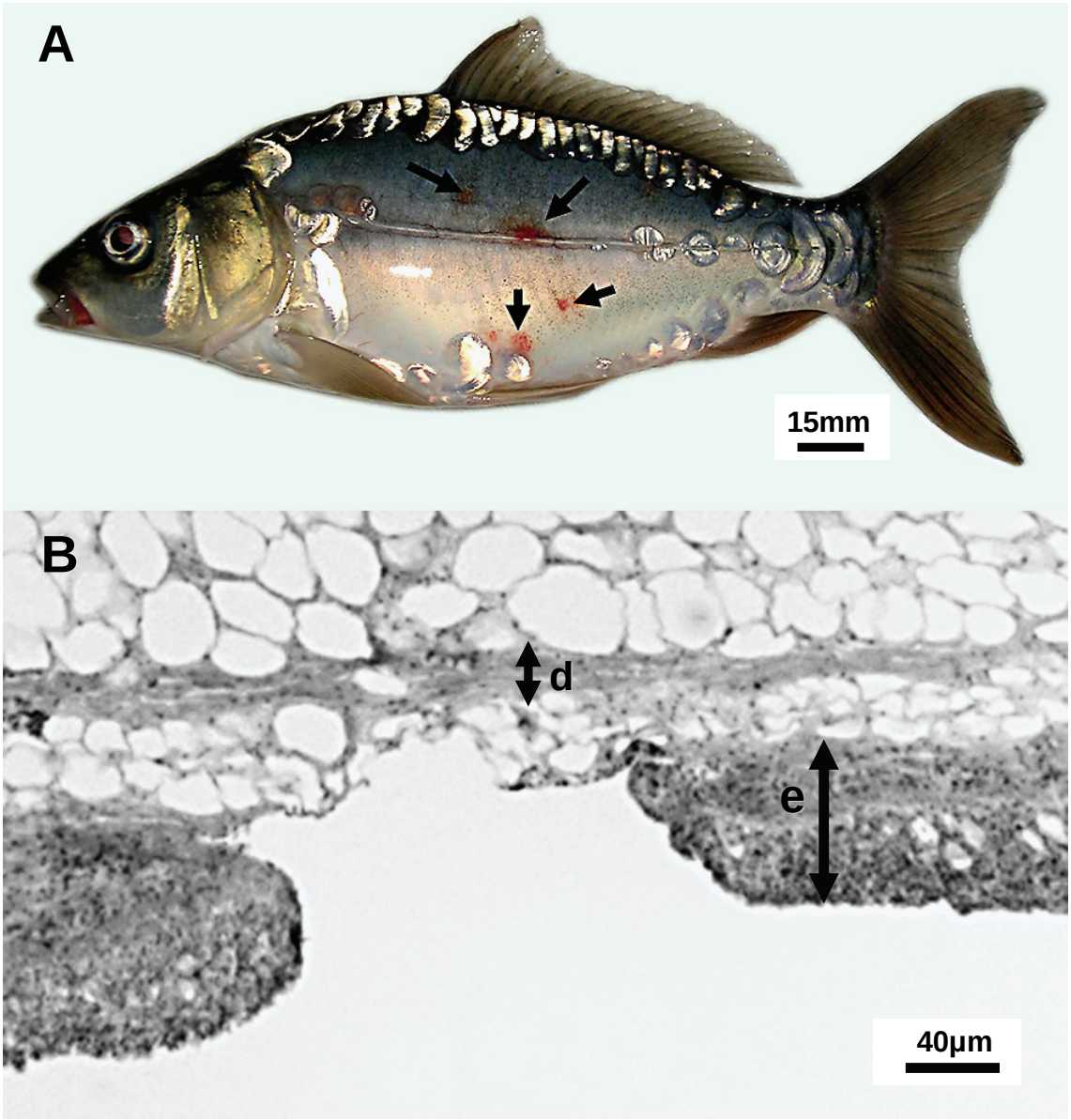


Fig 4. Juvenile common carp (*Cyprinus carpio*) 48hrs after infection with adult *Argulus japonicus* (A). Arrows indicate heamorrhagic lesions caused by feeding activities of the parasites. A section through the flank skin of a juvenile common carp (*Cyprinus carpio*) showing the crater-like wound inflicted by a feeding argulid (arrow) (B).

Sections through various regions of a fed parasites body in which gut diverticula are present, show that the various gut parts contain substances assumed to be the parasites last meal. TEM (Fig. 3E), clearly shows the presence of nucleated cells which were identified as erythrocytes from a fish.

Juvenile carp that had been infected for up to 48 hrs exhibited prominent red lesions on their skin (Fig. 4A). These lesions appeared haemorrhagic and were often associated with attached parasites. Microscopical examination of these lesions revealed craters in the skin of

the fish at the sites where parasites had been feeding (Fig. 4B). These craters penetrated as far as the dermis (stratum compactum) and infiltration of leukocytes into tissues surrounding the wounds was observed.

Discussion

Branchiuran lice have undergone major modifications of the mandibles and associated feeding appendages. Mandibles typically occur as a pair of transversely toothed hooks between the labium and labrum and this indeed is the case for specimens studied by us. These mandibles are located just inside the entrance to the buccal cavity forming part of the specialised proboscis (McLaughlin 1982). The mechanism of feeding in argulids has been described (e.g. van Duijn 1956; Kabata 1985; Overstreet *et al*., 1993; Lester & Roubal 1995) but there is some controversy concerning the facts. Much of this controversy relates to the function of the pre-oral stylet, an organ unique to species of *Argulus* and *Dipteropeltis.* Some authors are of the opinion that argulids use the pre-oral, retractable stylet to inject toxic substances into their host creating a localised inflammatory response with sub-cutaneous haemorrhaging. Gresty *et al*. (1993) also demonstrated that this organ was used to inject substances into the host rather than to withdraw fluids as was believed by other authors, e.g. van Duijn (1956) and Kabata (1970). The parasite then likely uses its serrated mandibles, which can be everted, to tear a hole in the epidermal layers to gain access to the host's blood. Whole blood (including erythrocytes) is then sucked up through a proboscis-like mouth tube, which, according to Kearn (2004), superficially resembles the mouth tube of a siphonostomatoid copepod.

Several authors have suggested that *Argulus* is haematophagous (see Walker *et al*., 2004 and Kearn, 2004 and referencs therein), but Kabata (1970) stated that red blood cells have not been recorded in the gut of *Argulus*. Our observations revealed the presence of large numbers of nucleated erythrocytes in the gut of adult *A. japonicus* just 2 hrs after feeding, and strongly indicate that whole blood forms a significant part of the diet of these parasites. The extensively branched nature of the gut in *Argulus* spp. is comparable to that of leeches, which are also designed to take up masses of blood (Wesenberg-Lund 1939). Whilst the precise function of the labial spines and pre-oral stylet has not been elucidated by this study we anticipate that future studies will demonstrate the presence of an anticoagulant aiding the hematophagous habits of *A. japonicus*. Certainly previous authors have described glands associated with pre-oral stylet and mouthparts which may produce such substances (Wilson 1903; Wesenberg-Lund 1939; Gresty *et al*. 1993; Kearn 2004).

In the case of larval *A. japonicus* the ingestion of whole blood (including erythrocytes) is unlikely. Erythrocytes from carp are typically around 12-13μm in the longest diameter (Imagawa, Hashioto, Kitagawa, Kon, Kudo & Sugimura 1989). Our observations regarding the aperture size of the opening to the parasites buccal cavity demonstrate that it is not large enough to allow the ingestion of intact red blood cells. In addition, no erythrocytes were observed in squash preparations of larval parasites. According to Tam & Avenant-Oldewage (2006) the sizes of the larval *A. japonicus* proboscis (approximately 50μm in length) and mandibular blades (approximately 15μm in length), located inside the proboscis, are probably insufficient to penetrate down to the blood vessels located in the dermis of a cyprinid host, due to the thickness of its epidermis (thickness = approximately 100μm). This probably accounts for the absence of erythrocytes in larval *A. japonicus*. Tam & Avenant Oldewage (2006) state that the food of larval *A. japonicus* appeared to consist of host mucous and epithelial cells and as with our study host erythrocytes were not observed.

Shimura & Inoue (1984) injected an extract derived from *Argulus coregoni* into rainbow trout (*Oncorhynchus mykiss*) and found that it induced a haemorrhagic response which would facilitate haematophagy by *A. coregoni.* Forlenza, Walker, de Vries, Wendelaar Bonga & Wiegertjes (2008) demonstrated an inflammatory response in carp skin following infection with *A. japonicus*. A similar haemorrhagic response was observed in carp in this study between 24 and 48 hrs post-infection suggesting that, like its relative *A. coregoni*, *A. japonicus* utilizes chemical secretions to facilitate feeding.

Blood feeding parasites have long been a topic of interest in many biological sub-disciplines, from the medical sciences to animal physiology and ecology. The reasons for studying animals that show this feeding behaviour are diverse and range from improving our understanding the importance of haematophagous animals as vectors for important diseases of farmed animals and humans (e.g. SVCV – Ahne, 1985; Malaria – Prevot, 2003; Lyme disease – Randolph, 1998) to raising our insights into role of these animals within ecosystems e.g. host population control. Kabata (1985) stated that aquaculturists should pay particular attention to the control of blood sucking parasites due to their potential as vectors for other serious pathogens (e.g. spring viraemia of carp virus, infectious pancreatic necrosis, bacterial hemorrhagic septicaemia). *A. japonicus* can now be confirmed as being within this important group of 'blood-suckers' and we believe that other argulids will also be confirmed as blood feeders in the future. However, Covich and Thorp (2001) suggest that blood feeding is likely to be species-specific among branchiurans.

Acknowledgements

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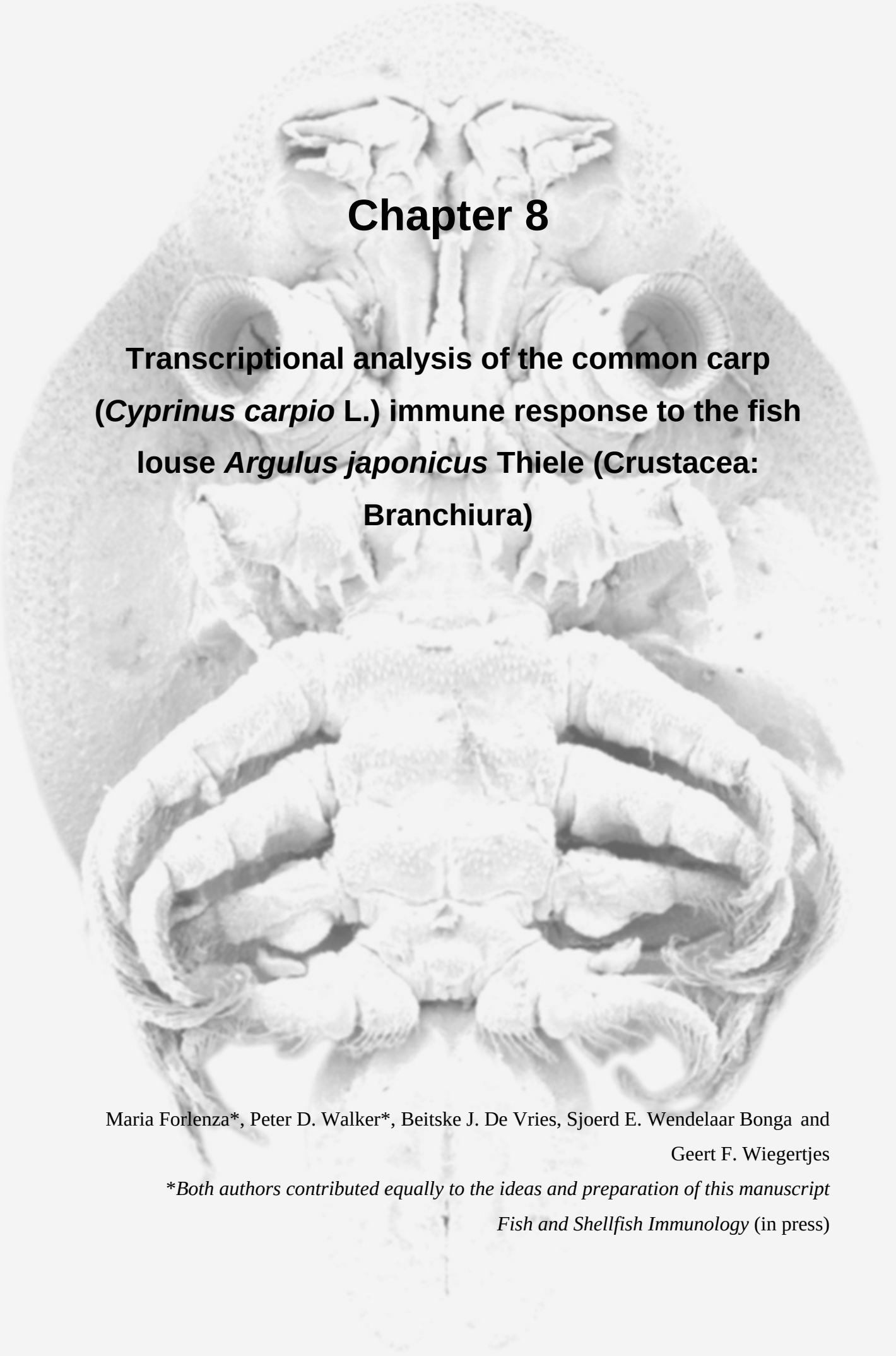

Chapter 8

Transcriptional analysis of the common carp (*Cyprinus carpio* L.) immune response to the fish louse *Argulus japonicus* Thiele (Crustacea: Branchiura)

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Abstract

In the present study we investigated changes in transcription levels of a panel of selected immune relevant genes in peripheral blood leukocytes (PBL) and skin samples collected from carp exposed to larval *Argulus japonicus.* We show that in skin, the up-regulation of gene transcription of the chemokine CXCa, and to a lesser extent that of the chemokine receptor CXCR1 and the cytokine TNFα, are good indicators of parasite-induced skin damage at 2 days post-parasite exposure. Up-regulation of gene transcription corresponded well with an increase in leukocyte, probably neutrophilic granulocyte, numbers in skin samples collected at the sites of infection. We show that time-point controls are essential when studying gene expression, especially in peripheral blood leukocytes (PBL). In addition, we demonstrate that non-infected control samples isolated from the skin of infected fish are suitable autologous controls, at least until after larval *A. japonicus* have undergone their first moult and begun to demonstrate increased mobility over their host's integument. The observed results are indicative of *A. japonicus* affecting the skin as a whole organ, particularly after the parasite's first moult, a phenomenon which has a great impact on correct skin sampling for RNA isolation.

Introduction

Parasite-induced damage and disease in fish is currently receiving a more intensive research focus from the scientific community. Fish lice have been shown to be a threat to farmed fish over countless decades and, more recently, to wild fish stocks. Sea lice, particularly those from the genus *Caligus* and *Lepeophtheirus*, have undergone intensive study as organisms in their own right (Johnson and Albright, 1991; Boxshall and Defaye, 1993; Krkošek *et al*., 2004) and over the last few decades the interactions between these animals and their hosts has also been the subject of several scientific investigations (Johnson and Albright, 1991; Nolan *et al*., 2000; Krkošek *et al*., 2004; Wells *et al*., 2006). However, certain groups of parasites, whilst economically important, have not been intensively studied with regard to the effects they have on their fish hosts.

Argulus spp. are typically regarded as generalist parasites and have been recorded from practically every species of fish occurring in the same habitat (Walker *et al*., 2004 and references therein). Occupying a similar niche to sea lice, the generalist freshwater lice from the genus *Argulus* have received a considerable amount of attention concerning their life cycle, morphology and ecology. However, the effects they have on their hosts are still poorly understood. Some of the most recent work has examined physiological and ultrastructural changes associated with stress induced by this parasitic organism (Nolan *et al*., 2000; van der Salm *et al*., 2000). However, despite the economic importance of this group of pathogens (in terms of their deleterious effects on fish stocks (Walker *et al*., 2004)), few studies have addressed the immune response of fish to an infestation with these parasites. To combat these pathogens successfully it is vital to gain a comprehensive understanding of the natural defence mechanisms employed by fish (Walker *et al*., 2004), for example by gene transcription studies in tissue samples of infected fish.

Skin is an essential protective barrier for fish and functions as a first line of defense against infectious microbes from the aqueous environment. The cell composition of the epidermis is well known and in common carp (*Cyprinus carpio* L.) the epidermis consists mainly of filament cells, mucous cells, club cells and an upper layer of pavement cells. The dermis contains chromatophores and melanophores (Iger *et al*., 1994). In the epidermis and dermis of healthy fish, small numbers of lymphocytes and macrophages can be found (Iger *et al*., 1994). Nevertheless, limited information is available for this organ with regard to immune response mechanisms and associated gene transcription. A few recent studies have shown regulation of immune gene transcription in fish skin following infection with ectoparasites (Lindenstrom *et al*., 2003; Lindenstrom *et al*., 2004; Sigh *et al*., 2004a,b; Gonzales *et al*.,

2007). Further, the blood is essential for mounting a rapid immune response, for example by transportation of the relevant leukocytes, often neutrophilc granulocytes, to the site of inflammation.

In the present study we investigated changes in gene transcription in peripheral blood leukocytes (PBL) and skin of common carp (*C. carpio*) infected with larval stages of the ectoparasite *Argulus japonicus*. Larval stages were chosen over adult stages, not only due to the fact that larger numbers were obtainable, resulting in a potentially higher number of attached parasites per fish, but also because larval lice appear to migrate over the surface of their host to a much lesser degree than their adult counterparts (unpublished observation). This will increase the probability of a synchronized timing of the immune response resulting in reduced variation measured between samples. We collected time-point control samples from non-infected fish for the gene transcription studies and, in addition, for the studies on the host skin we included autologous time-point control samples corresponding to non-infected spots isolated from infected fish. We also performed a histological examination of sites of infection to examine the putative contribution of migrating leukocytes to changes in gene transcription.

Materials and methods

Animals

European common carp (*Cyprinus carpio carpio* L.) were bred in the central fish facility of Wageningen University, The Netherlands, and raised in recirculating UV-treated water at 23^{o}C (± 1^{o}C) and with a 12:12 light:dark photoperiod in the central fish facility of Radboud University Nijmegen and fed pelleted dry food (Trouvit, Nutreco) daily. R3R8, carp which are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain), were used (Irnazarow, 1995). Fish were divided over 8 aquaria, each containing 10 fish. All studies were performed with the approval of the animal experimental committee of Radboud University Nijmegen.

Parasites

A population of *Argulus japonicus* was maintained on ‘stock’ carp (approximately 1000g fish). Infestation intensities typically varied from 10-30 lice per fish. Eggs were deposited on the glass sides and bottoms of the aquaria. Host fish were monitored regularly and parasite eggs were removed to control parasite numbers when infestation intensities appeared to be too

heavy as indicated by host condition and host-behavioural changes e.g. lethargy and loss of appetite.

Parasite collection

Adult *A. japonicus* were collected from stock carp that had been anaesthetised in a 2-phenoxyethanol (Sigma-Aldrich, St Louis, MO, USA) solution (dilution = 1:1000). Parasites were subsequently removed from all fish using a set of blunt forceps and then held in beakers containing tap water (non-chlorinated) at 23^{o}C for 48 hrs. During this time any eggs deposited by lice were collected and incubated in tap water at 23^{o}C with daily refreshment of the water. Upon hatching larval lice were held in groups of 150 individuals/beaker under identical conditions as for the eggs/adult lice for 24 hrs prior to the start of the experiment. This increased the likelihood that stored food reserves were fully diminished prior to the start of the experiment (Tam and Avenant-Oldewage, 2006), increasing the likelihood that parasites would immediately seek out and attach to a fish.

***Infection of carp with* Argulus japonicus**

To infect the carp, the beakers of water containing larval lice were emptied into 5 (randomly selected) of the 8 experimental aquaria. The same amounts of tap water was also poured into each of the 'control', non-infected tanks. This marked the time zero (T0) time point. One fish was then removed from each of the tanks at T0, 10 h, 24 h (1 day), 48 h (2 days), 72 h (3 days) and 6 days post-infection. Upon removal, fish were irreversibly anaesthetized in 2-phenoxyethanol (dilution = 1:500) and subsequently weighed, measured (standard length) and the number of attached parasites recorded.

Peripheral blood leukocytes (PBL) and skin isolation

Blood was collected via puncture of the caudal vessel and diluted 1:1 with cRPMI (RPMI 1640; Cambrex, Verviers, Belgium; adjusted to 270 mOsmol kg^{-1}) containing 50 IU/ml of heparin (Leo Pharmaceutical products, Weesp, The Netherlands). After centrifugation at 600 g for 10 min, the buffy-coat containing leukocytes was collected and layered on 5 ml of Ficoll-Paque™ Plus (Amersham Biosciences, Uppsala, Sweden). Following subsequent centrifugation at 800 g for 25 min, the PBL at the interface were collected and washed three times with cRPMI. Cell pellets were collected, immediately snap frozen in liquid nitrogen and stored at -80ºC until used for RNA isolation.

Several 5 x 5 mm samples of skin were carefully removed from the ventral region of the flanks of uninfected (control) and infected fish. For infected fish, samples were taken from sites of parasite attachment/feeding (infected spots) and also from sites distant to the sites of infection (autologous controls). The number of infected spots differed between individual fish. Skin samples were then immediately snap frozen in liquid nitrogen and stored at -80ºC until use or immersed in Bouin's fixative for subsequent processing and histological analysis.

Histological analysis

Skin samples for histological analyses were fixed for a minimum of 24 h in Bouin's fixative. Samples were subsequently dehydrated through a graded series of ethanol and embedded in paraffin. Five-µm sections were mounted on gelatinized glass slides and dried overnight in an oven at 40^{o}C. Slides were then stained with hematoxylin and eosin and examined microscopically for evidence of parasite-induced damage and host inflammatory responses (i.e. infiltration of leukocytes).

RNA isolation and quantification

For Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) analysis total RNA was isolated from PBL and skin using the RNAeasy Mini Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. On-column DN*ase* treatment with the RN*ase*-free DN*ase* set (Qiagen) was also included.

RNA was isolated, separately from 3 skin pieces from each of the three non-infected controls or from 5 pieces (autologous controls) and 1-4 pieces (infected spots) from each of the five infected fish. RNA isolated from more pieces from the same individual was never pooled and each piece was handled and analyzed separately.

The concentration of RNA was measured spectrophotometrically (GeneQuant, Pharmacia Biotech) at OD_{260nm} and the purity determined as the OD_{260nm}/OD_{280nm} ratio with expected values between 1.8 and 2.0. The integrity of RNA was determined by electrophoresis on 1% agarose gel containing 0.5 µg/ml ethidium bromide at 100 V. Total RNA was stored at -80ºC until further use.

cDNA Synthesis

Prior to cDNA synthesis, a second DN*ase* treatment was performed using DN*ase* I, Amplification Grade (Invitrogen, Breda, The Netherlands). Briefly, 1 µg of RNA from each sample was combined with 1 µl 10x DN*ase* reaction buffer and 1U DN*ase* I up to a final

volume of 10 µl, mixed and incubated at RT for 15 min, followed by inactivation of DN*ase* I by adding 1 µl of 25 mM EDTA. Synthesis of cDNA was performed with Invitrogen's Superscript™ III First Strand Synthesis Systems for RT-PCR, according to the manufacturer's instructions. Briefly, DN*ase* I-treated RNA samples (11 µl) were mixed with 5x first strand buffer, 300 ng random primers, 10 mM dNTPs, 0.1 M DTT, 10 U RN*ase* inhibitor, and 200 U Superscript III Reverse Transcriptase (Invitrogen) up to a final volume of 20 µl. The mixture was incubated at 37ºC for 60 min followed by an inactivation step at 70ºC for 15 min. A non-reverse transcriptase control was included for each sample. Before use as template in RT-qPCR experiments, the cDNA was further diluted 25 times in nuclease-free water.

Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR using SYBR Green I technology was performed using a Rotor-Gene™ 2000 (Corbett Research, Mortlake, Sydney, Australia) with the Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry. The primers used for RT-qPCR are listed in Table 1. PCR conditions were optimized by analyzing the melting curves of the products and product specificity was assessed by analysis on a 1% agarose gel. Master-mix for each PCR run was prepared as follows: 0.32 µl of water, 0.84 µl of each primer (5 µM), 7 µl Master SYBR Green I mix. Finally, 5 µl of diluted cDNA was added to 9 µl of master mix and transferred to a 0.1 ml tube. The following amplification program was used: after 15 min of denaturation at 95ºC, 40 cycles of RT-qPCR with three-step amplification were performed - 15 s at 95ºC for denaturation, 30 s at 60ºC for annealing and 30 s at 72ºC for elongation followed by a final holding step of 1 min at 60ºC. A melting step was then performed with continuous fluorescence acquisition starting at 60ºC with a rate of 1ºC/5s up to 99ºC to determine the amplification specificity. In all cases, the amplifications were specific and no amplification was observed in negative controls (non-template control and non-reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analyzed using Rotor-Gene version 6.0.21 software and exported to Microsoft Excel. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software. Briefly, the E for each primer set was recorded per sample and an average E (E_A) was then calculated for each primer set. The relative expression ratio (R) of a target gene was calculated based on the E_A and the Ct deviation of sample versus control, and expressed in comparison to a reference gene (Pfaffl, 2001; Tichopad *et al*., 2003). Gene expression analysis in skin tissue was

performed for each sample separately, even for those isolated from the same individual. The R in each treated sample (autologous control and infected spot) was calculated relative to a total of nine non-infected pieces collected from three time-point controls. For analysis in PBL, controls at time point zero (n=5) were used for relative expression analysis. Only at the end mean R of each sample in each time point was calculated and used for statistical analysis. The 40S ribosomal protein S11 was used as an internal reference gene.

Table 1. Primers used in RT-qPCR gene expression analysis.

Primer	Sequence 5' → 3'	Product (bp)	Acc no
qIL-1β.FW	ACGCCACCAAGAGCCTTTTA		
qIL-1β.RV	GCAGCCCATATTTGGTCAGA	69	AJ245635
qTNFα.FW	GCTGTCTGCTTCACGCTCAA		
qTNFα.RV	CCTTGGAAGTGACATTTGCTTTT	106	AJ311800
qCXCa.FW	CTGGGATTCCTGACCATTGGT		
qCXCa.RV	GTTGGCTCTCTGTTTCAATGCA	88	AJ421443
qCXCRI.FW	GCAAATTGGTTAGCCTGGTGA		
qCXCRI.RV	AGGCGACTCCACTGCACAA	144	AB010468
qIL-10.FW	CGCCAGCATAAAGAACTCGT		
qIL-10.RV	TGCCAAATACTGCTCGATGT	103	AB110780
q40S.FW	CCGTGGGTGACATCGTTACA		
q40S.RV	TCAGGACATTGAACCTCACTGTCT	69	AB012087

Statistics

Relative expression ratios (R) were calculated as described above. Transformed values (ln(R)) were used for statistical analysis in SPSS Software (version 15.0). Homogeneity of variance was analysed using the Levene's test. Significant differences ($P<0.05$) were determined by a two-way ANOVA followed by a Sidak test. In case of unequal variances between groups, the two-way ANOVA was followed by a Games-Howell test.

Results

General observations

Larval parasites were found attached to fish just 2 h post-infection. Infection was confirmed for all carp exposed to the parasites. However, the number of attached lice/fish was low and varied between individual fish (mean = 3.8 ± 3.1 S.D.) in all tanks. Infection intensities did not exhibit a time related change in parasite numbers. No significant behavioral changes in the host fish were observed at any time during the course of the experiment. The first gross pathological signs of infection were visible as red spots/lesions on the surface of infected fish and appeared between 24 and 48 h post-infection (p.i.). These lesions varied in size up to a maximum of 6 mm across. Parasites were only observed as larval stages until approximately day 5 post-infection, when the vast majority of observed parasites had undergone their first moult, becoming juveniles.

Gene expression analysis in PBL

We investigated the kinetics of expression of several immune-relevant genes in PBL of non-infected and fish infected with the ectoparasite *A. japonicus.* On day 1 to 2 p.i., a significant ($P<0.05$) up-regulation of IL-1β (6-fold), TNFα (3.2 fold) and of the chemokine receptor CXCR1 (3.5-fold) but not CXCa was observed when compared to the control at time point zero (T0, Fig. 1). However, no significant differences were observed when the gene expression levels were compared with the respective time point controls. In fact, the same genes were upregulated in PBL samples from individual time point controls, possibly owing to unknown environmental stimuli. On day 6 p.i. both IL-1β and IL-10 transcript levels were significantly up-regulated when compared to the control at time point zero (Fig. 1) but, again, not when compared to the time point controls.

Gene expression analysis in skin

To investigate not only the local immune response at the site of infection but also a more generalized response which might affect the skin as a whole, we analyzed the kinetics of expression of several immune relevant genes in samples collected from both infected spots and non-infected spots (autologous controls) of infected fish. Kinetics of expression were compared to those observed in samples collected from non-infected fish at each individual time point. For clarity, since infected spots were not visible until 24 h, the 10 h time point was

excluded from the analysis. Two days post-infection CXCa showed a significant up-regulation when

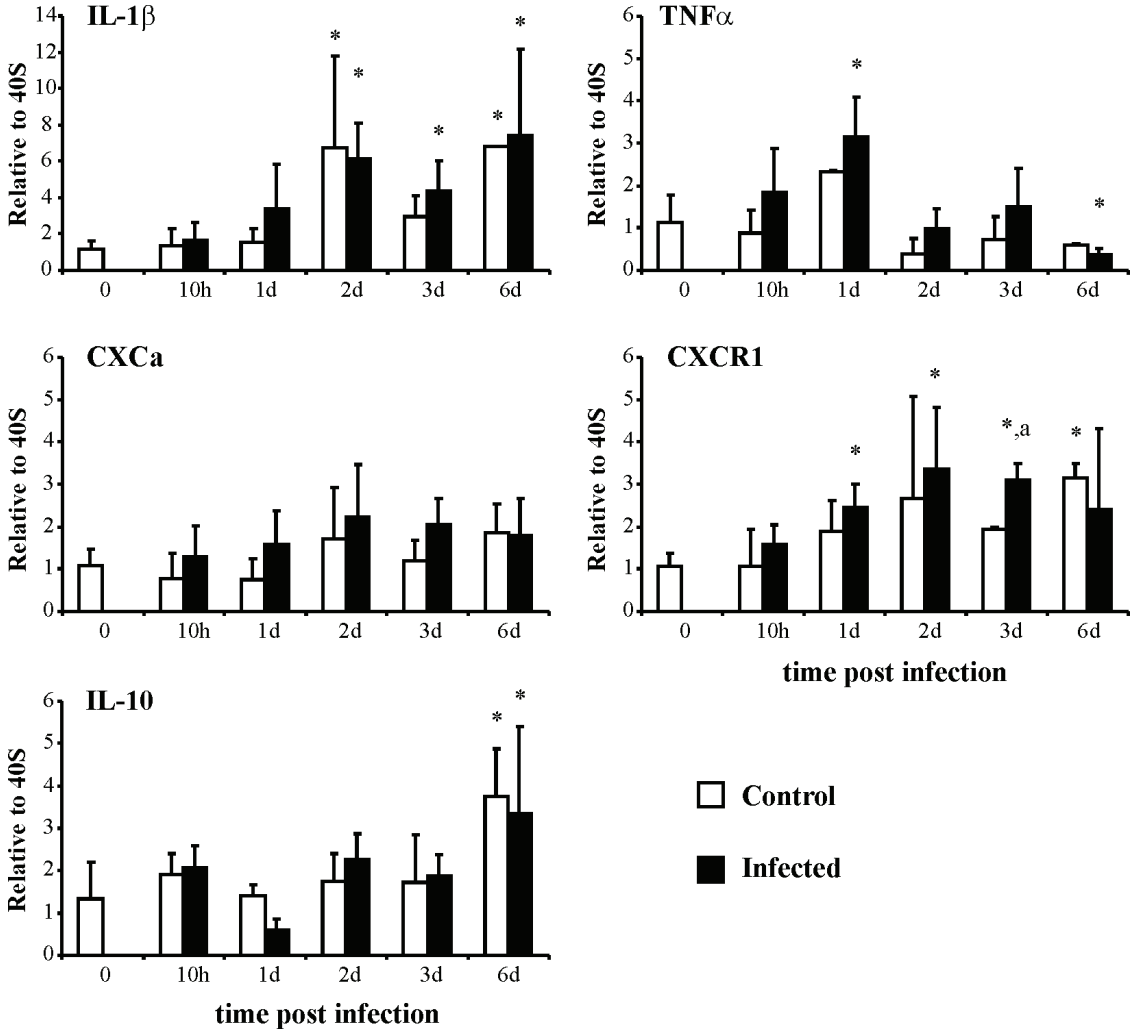

Figure 1. Kinetics of gene expression in PBL after *Argulus japonicus* infection of common carp (*Cyprinus carpio* L.). Fish were exposed to 150 larval parasites/tank by bath challenge. PBL from non-infected and infected fish were collected at different time points post-infection. Expression was determined by Real-Time quantitative PCR and expressed relative to S11 protein of the 40S subunit. Data represent mean values of: n=5, 0h controls; n=5, infected fish; n=3, time-point controls (± SD). Symbol '*' represents a significant difference as compared to non infected controls at time point zero. Symbol 'a' indicates a significant difference as compared to the non infected time-point control.

compared to the autologous controls and also when compared to the non-infected time-point control (Fig. 2). TNFα and CXCR1 transcription levels were also elevated at 2 days p.i. but this difference was significant only when compared to the non-infected autologous control. At day 6 p.i., TNFα, CXCa and IL-10 transcription levels were significantly up-regulated in skin samples collected from infected spots when compared to the non-infected time-point control. At the same time point, only for TNFα and CXCa, significantly elevated transcription levels

were observed in the autologous control samples when compared to the respective non-infected time-point control.

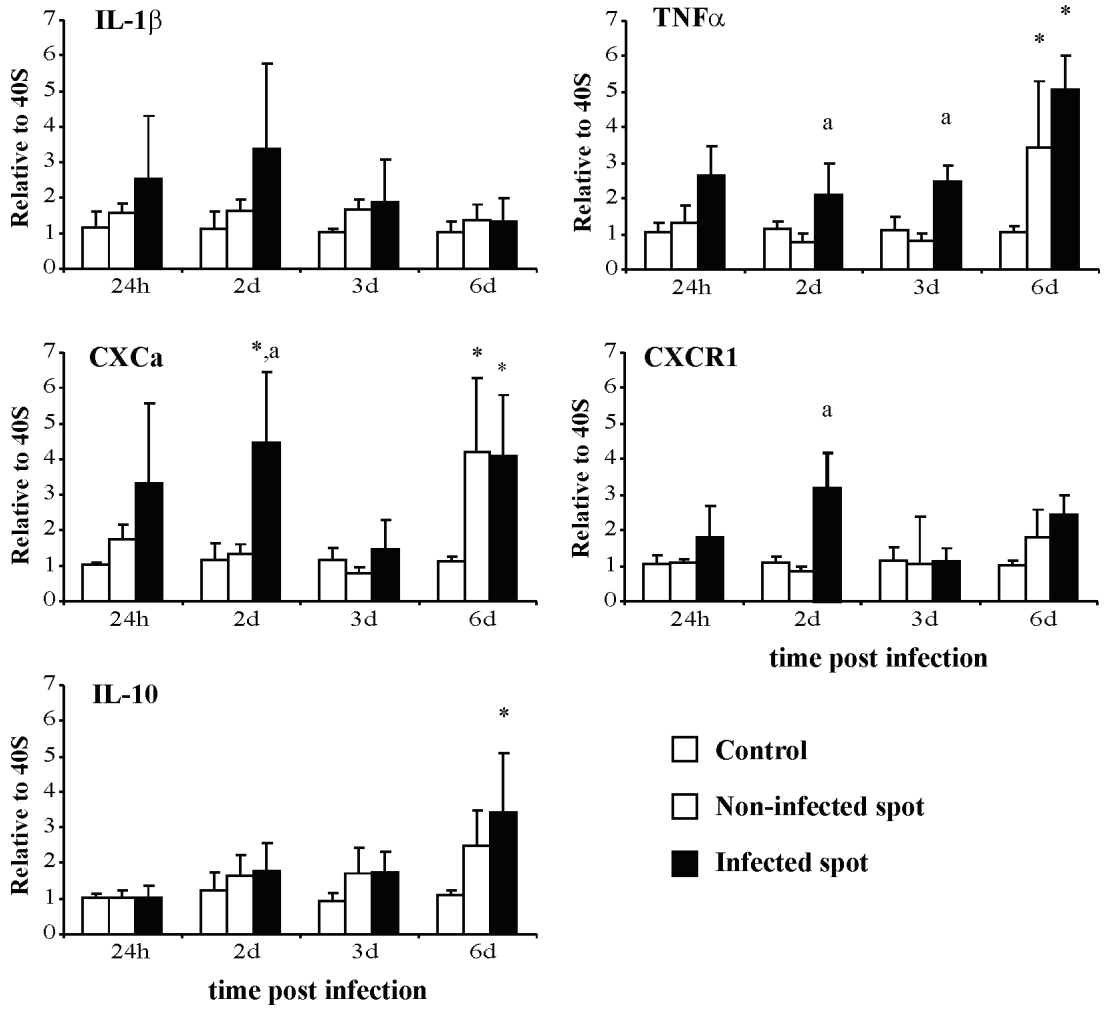

Figure 2. Kinetics of gene expression in skin after *Argulus japonicus* infection of common carp (*Cyprinus carpio* L.). Fish were exposed to 150 larval parasites/tank by bath challenge. Skin samples were collected at different time points post-infection from non-infected fish, non-infected spots of infected fish (autologous control) and infected spots from infected fish. Expression was determined by Real-Time quantitative PCR and expressed relative to S11 protein of the 40S subunit. Data represent mean values of n=5 infected fish and n=3 time-point controls (± SD). Symbol '*' represents a significant difference as compared to the non-infected time-point control. Symbol 'a' indicates a significant difference as compared to the autologous control.

Histological analysis

To investigate whether chemokine (CXCa) up-regulation of gene transcription could be related to increased transcription activity in leukocytes already present in the skin or to an increased number of leukocytes in the skin following infection, we performed a histological analysis of the skin samples. Figure 3B shows a massive infiltration by leukocytes at the site of infection. A large amount of inflammatory infiltrate can be observed in both the mildly

hyperplastic epidermal and dermal tissue and also throughout the whole subcutaneous fatty layer. A very low number of leukocytes could be observed in control skin samples (Fig. 3A).

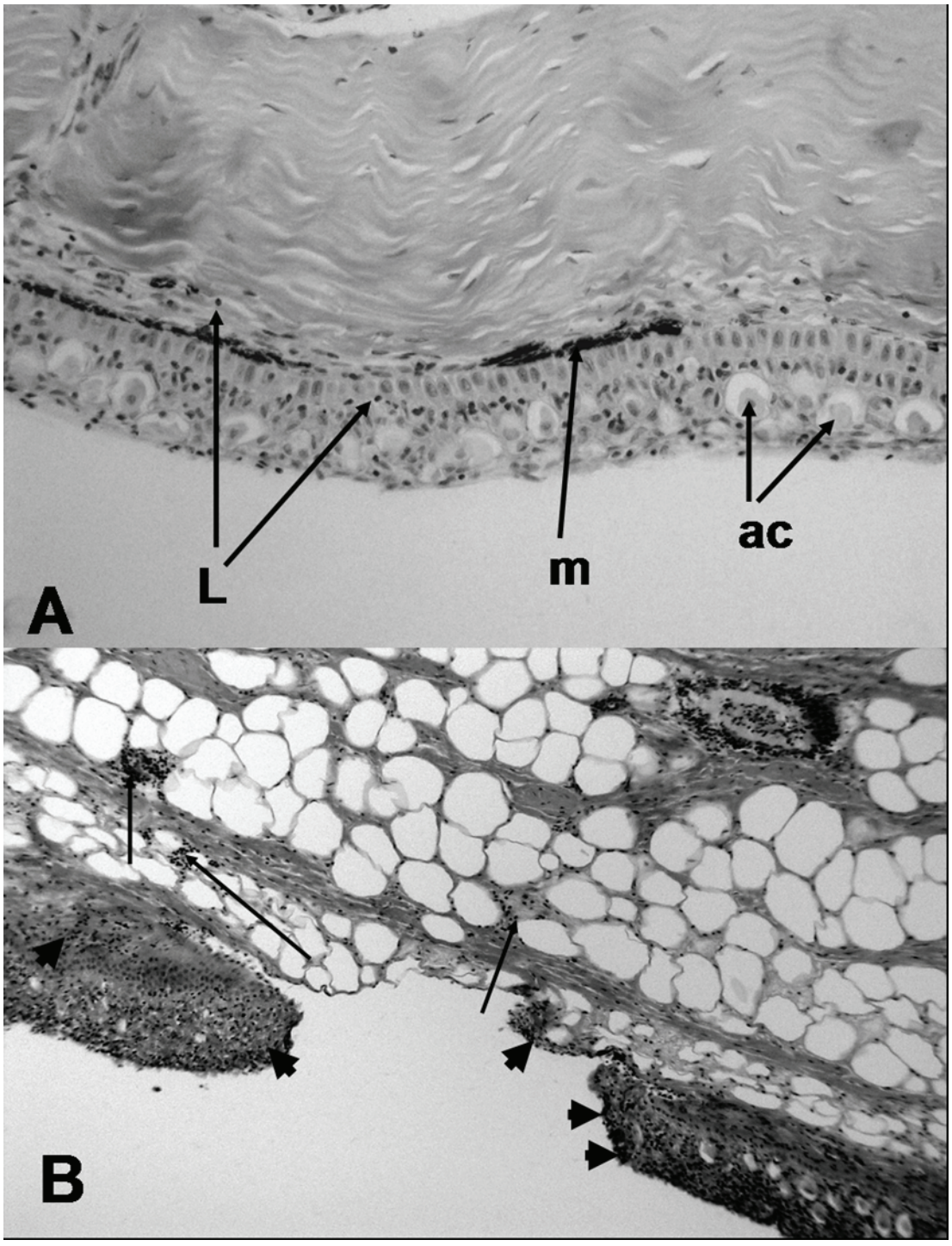

Figure 3. Histological analysis of carp skin. A) Skin sample from a control, non-infected fish. Note that there are only a few scattered leukocytes and no inflammation of the tissues (magnification = 1000x), L = leukocytes, m = melanophores and ac = club cells. B) Skin sample from *A. japonicus* infected fish. Infected spot collected 2d p.i., at site of parasite feeding. A crater can be observed in the epidermis and apical part of the dermis. A high degree of leukocyte infiltration can be observed in both the mildly hyperplastic epidermal and dermal tissue (thick arrows) and a large number of leukocytes can also be seen dispersed throughout the subcutaneous fatty layer (thin arrows). Note the leukocytes dispersing from the blood vessel at the top right of the image (magnification = 300x).

Discussion

We investigated the immune response of common carp to freshwater lice from the genus *Argulus*. The most pronounced upregulation of gene transcription was observed for the chemokine CXCa in the skin at 1-2 days post-infection with larval *Argulus japonicus*. At the same time a massive infiltration by leukocytes, most likely neutrophilic granulocytes, at the site of infection could be observed in histological sections of the skin. At day 6 post-infection immune gene transcription was up-regulated not only in infected skin but also, although to a lesser extent, in autologous skin sample controls collected from non-infected spots of infected fish. This suggests that the response to *A. japonicus* larval stages is initially restricted to the site of infection but is extended to a generalised response throughout the skin as a whole organ at a later stage of the infection. In PBL, transcription levels of the investigated genes varied greatly in control fish, emphasizing the importance of time-point control samples for gene transcription studies.

During our experiment, the first visible red, inflamed spots were not observed until approximately 2 days post-infection, despite the attachment of several larval lice to the skin of exposed fish as early as 2h post-infection. In other experiments, the first notable response to argulid infestations, observed as small red areas (up to several mm in diameter), has been noted even within a few hours post-infection (Walker *et al*., 2004). Although we could confirm infection for each individual fish, the infection pressure in our experiment was relatively mild as shown by a mean infection intensity of 3.8 attached parasites per fish. Further, we chose to investigate the immune response to larval rather than juvenile or adult lice. Adult argulids are quite mobile being able to glide over the surface of their hosts with relative ease using their maxillary suckers (Kearn, 2004). This can result in multiple sites of infection on the same host being caused by one parasite at different times. However, larval stages tend to be less mobile and remain relatively stationary, anchoring themselves to their hosts integument via the use of their hook-like 2nd antennae (Walker *et al*., 2004). *Argulus* spp. cause direct damage to the fish skin through their attachment and feeding mechanisms although, typically, the craters do not penetrate much deeper than the epidermis (Lester and Roubal, 1995). Skin damage is the result of mechanical actions of the maxillary suckers in adult lice and hooks or spines in larval and juvenile stages and the sharp mandibles. In addition, damage results from various toxins or digestive enzymes secreted via the pre-oral stylet and labial spines (Shimura and Inoue, 1984).

Two days post-infection, levels of CXCa transcripts were up-regulated in skin samples collected at the sites of infection (infected spots), and were significantly different from the

levels measured in skin isolated not only from non-infected control fish, but, also from autologous skin samples collected from non-infected spots of infected fish. At the same time point, TNFα and CXC receptor-1 (CXCR1) transcription were found to be up-regulated with respect to the autologous control. In comparison to the response observed 1-2 days post-infection, at 6 days post-infection a clear upregulation of CXCa, TNFα and, to a lesser extent, IL-10 transcripts was observed in skin samples collected from both infected and non-infected spot of the infected fish. These results indicate that whilst the immune response to *A. japonicus* larval stages (2 days post-infection) is restricted to the site of infection, the response to juvenile stages (6 days post-infection) is extended throughout the skin as a whole. For *A. japonicus*, in fact, a succession of moults takes place approximately every 5 days, depending on the ambient temperature (Fryer, 1982). It is likely, also during our experiment, that after 5 days *A. japonicus* larval stages started to moult and migrate over the skin of the fish host. This would explain why the change in gene expression profile was similar, although somewhat smaller, between autologous skin sample controls and infected skin samples. The observed results are suggestive of migrating *A. japonicus* affecting the skin as a whole organ.

In contrast to the results obtained after the analysis of the skin samples, changes in gene expression in samples collected from PBL were found to be significantly different only when compared to the controls at time zero, indicating the vital importance of time point controls when employing RT-qPCR analyses of gene expression, at least in PBL. The same PBL samples were used for a flow cytometric analysis (unpublished data) using antibodies directed against carp macrophages (Weyts *et al*., 1997) and granulocytes (Nakayasu *et al*., 1998). In this flow cytometer experiment we found a very high fish-to-fish variation not only between infected but also between non-infected individuals, confirming the importance of proper time-point controls, especially when analysing PBL.

In the present study we show an up-regulation of the chemokine CXCa in the skin of common carp exposed to larval *A. japonicus* at 1-2 days post-infection. Although at the same time-point transcription of the chemokine receptor CXCR1 and of the cytokines IL-1β and TNFα seemed to be upregulated, these changes were not significantly different from the non-infected controls. Although not significant, probably due to the large individual variation, these changes might reflect a biologically relevant phenomenon. The same immune genes (CXCa, CXCR1, IL-1β), in fact, were up-regulated in carp skin at 1.5-2 days after infection with the ectoparasite *Ichthyophthirius multifiliis* (Gonzalez *et al*., 2007). The chemokine CXCa and the chemokine receptor, CXCR1, have been implicated as factors stimulating the migration of neutrophilic granulocytes towards sites of infection (Huising *et al*., 2003). We

noticed an increase in number of infiltrating leukocytes, most likely neutrophilic granulocytes, at sites of skin damage in histological sections of carp skin infected by *A. japonicus*. This would suggest a correlation between up-regulation of CXCa (CXR1) and the migration of neutrophilic granulocytes. Recently, a study in carp skin on the changes in gene transcription induced purely by mechanical injury reported an up-regulation of the same set of genes: CXCa, CXCR1, IL-1β and to a lesser extent TNFα 2-3 h after injury (Gonzalez *et al*., 2007). In the latter study also migrating neutrophils were observed in histological sections of damaged skin. In general, the results of tissue damage are recognized at the cell level via receptor-mediated detection of intracellular proteins (alarmins) released by dead cells and, indeed, endogenous alarmins but exogenous pathogen-associated molecular patterns (PAMPs) also convey similar (immune) responses and can be considered subgroups of a larger set, the damage-associated molecular patterns (DAMPs) (Bianchi, 2007). The CXCa, CXCR1 and IL-1beta genes especially, seem to be part of a set of immune genes in fish that are commonly induced by DAMPs.

We aimed to design an optimal animal experiment by using a mild pathogen load mimicking a natural situation where both host and parasite are expected to co-exist and survive. We used time-point controls for both skin and PBL samples. These latter controls were shown to be essential since differences in transcription levels noted especially in the PBL samples were significant only when compared to the controls at time-point zero. In addition, we used autologous skin samples as controls for infected spots from the same fish, an approach which proved highly informative, especially at later time points. In conclusion, we demonstrate that in the skin of carp exposed to larval/juvenile *A. japonicus* up-regulation of gene transcription for the chemokine CXCa and to a lesser extent the chemokine receptor CXCR1 and the cytokines IL-1ß and TNFα are good indicators of parasite-induced skin damage. Up-regulation of gene transcription corresponded well with an increase in leukocyte numbers, possibly neutrophilic granulocytes, in skin samples collected at the sites of infection.

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Chapter 9

General Discussion

A detailed knowledge of fish parasites biology and ecology and the effects parasites have on their hosts is of huge importance, even more so today than in the past due to the ever increasing role of aquaculture and fisheries in supplying global protein demands. Parasites of fish cause high commercial losses in both the aquaculture (Menezes *et al*., 1990) and fisheries industries (Taylor *et al*., 2006; Krkošek *et al*., 2007) and can have serious socio-economic in both developing and developed countries (Barber *et al*., 2000).

The intimate relationship between parasites and there hosts have typically co-evolved over countless generations. Parasites are continually locked in an evolutionary arms race (Red Queen Hypothesis; see Woolhouse and Webster, 2000) with their hosts, each species constantly evolving new strategies to increase its fitness and gain an advantage over the other. Ultimately however a parasite must aim to have a minimum negative impact upon its host so that it does not directly, or indirectly, cause the death of the host, thus forcing the parasite to locate a new host or perish with its current one. Parasitology, the study of parasites and their habits, provides valuable information about how parasites live, reproduce, feed and interact with their hosts.

Aims

The aim of this PhD research project was to identify key areas in which information regarding the ecology, physiology and host-parasite interactions of *A. foliaceus* and *A. japonicus* was lacking and subsequently try through field observations and experimental investigations to bridge some of these gaps in our knowledge of these economically important parasites.

In order to realize precisely what was missing it was important to first gain a thorough understanding of what information was currently available. This was achieved by the compilation of an extensive literature review presented in Chapter 2, which reviews the majority of the available published material on these animals. From this review it became apparent that there were important topics where information was indeed lacking or contradictory. Much of the contradictory statements in the literature appeared to be the result of statements being made in the absence of sufficient scientific evidence to support the claims. Chapter 2 concludes by stating that “much of the basic knowledge regarding these organisms’ biology is still not fully understood and in many cases may prove vital to our understanding of the complex relationships between pathogens and their hosts”. The following synthesis will show how this thesis has contributed to the knowledge regarding *Argulus* spp. and their hosts. Like all scientific investigations however, it has also resulted in the generation of new

questions, confirming that the more we discover about a topic, the more we realize we have yet to discover.

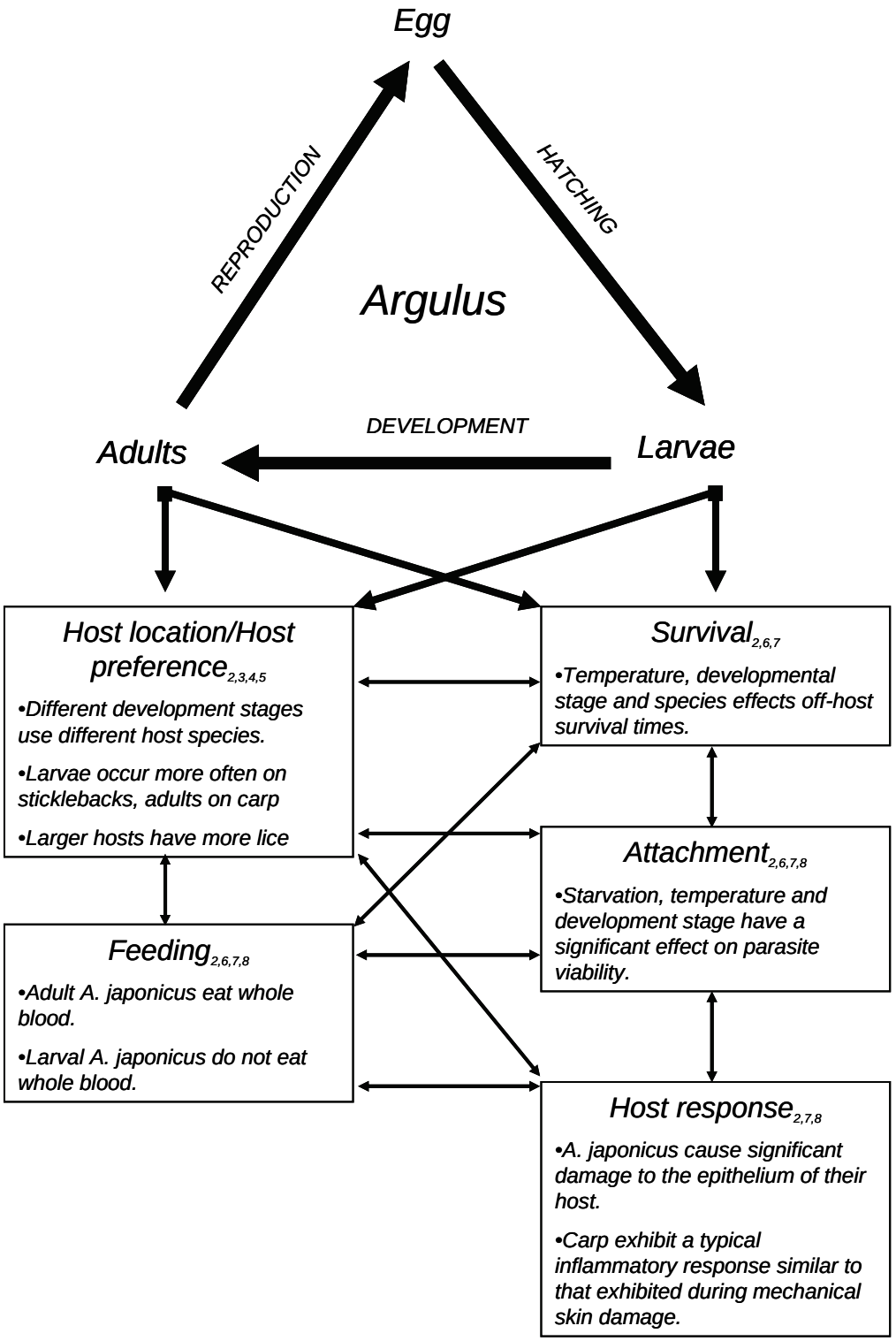

Figure 1. Schematic representation of the way the environment (both abiotic and biotic) influences the distribution of a generalised argulid via effects on its behaviour, ecology and physiology. Key aspects of the life history between larval hatching and adult maturation/reproduction and how these aspects are interrelated are shown. Numbers given in text boxes correspond with chapters in this thesis, where each of these aspects is addressed to some extent.

The diagram presented in the introductory chapter of this thesis (Chapter 1, Fig. 1) illustrates key topics addressed in subsequent chapters. This diagram is presented here in an amended form (Fig. 1) and the following discussion will aim to highlight how, through the various chapters in this thesis, we have increased our knowledge on these aspects of *Argulus* parasitology. The ways in which these aspects are inter-related will be discussed simultaneously.

Life Cycle

As illustrated in Fig. 1, upon hatching, the larval argulid is a free-swimming animal with a finite energy reserve. This parasite must survive (Survival) long enough to successfully locate a suitable host (Host location; Host preference). Once a potential host has been identified the larval parasite must attach to its host (Attachment) before it can begin feeding (Feeding). The feeding activities of this parasite cause damage to its host and this elicits various responses from the host including activation of stress and immune responses, and often includes behavioural changes such as flashing behaviour and leaping clear of the water to try and shed lice (Chapter 2; Wesenberg-Lund, 1982). As the larval parasite matures, it may switch hosts several times. Reasons for host switching are not clear but may include accidental dislodgement of the parasite, competition for a suitable attachment/feeding site or it may be some aspect of host-preference exhibited by the lice. Once the parasite becomes sexually mature it must locate a female in order to reproduce. Copulation typically occurs on a host fish (Chapter 2). In order to locate a suitable mate, parasites may be forced to abandon their hosts. Once a female has mated she must then also abandon her host to deposit her eggs on a suitable substrate.

The life cycle of an argulid includes a constant switching between on-host and off-host periods. Each time a parasite loses contact with its host it must therefore survive for an indefinite period of time off-host (Chapter 6) until it can successfully locate a new host (Chapters 2, 3, 4 and 5) upon which it can attach and feed (Chapters 2, 6, 7 and 8). Feeding activities of these lice will ultimately illicit a response in their hosts (Chapters 2, 7 and 8) which may result in unfavourable conditions for the parasite resulting in it changing location on the host or once again abandoning its host in search of a more preferable one (Chapters 2, 3, 4, 5 and 7). And so each time the cycle continues with the parasite constantly facing challenges relating to survival, host location, attachment, host preference, feeding and host responses.

Host location/Host preference

Q1) Are some fish species more likely to be infested with *A. foliaceus*?

A1) Yes.

It has been frequently stated that parasites from this genus show little or no host specificity (Chapter 2, 3, 4 and 5 and references therein). Studies by (among others) Kennedy (1974), Lamarre and Cochran (1992), Mikhhev *et al*. (1998),Evans and Mathews (2000), Pasternak *et al*. (2000) and Mikheev *et al*. (2004), suggest that whilst species such as *A. foliaceus*, *A. japonicus* and *A. coregoni* may be capable of infecting practically any freshwater fish they encounter, various factors may result in some fish being more prone to infestation than others. Despite the countless records of *A. foliaceus* from a multitude of different host species there appeared to be a lack of studies regarding the epidemiology of this parasite within natural systems.

Individual species seem to have an important influence on infection levels (Chapter 3). We concluded on the basis of evidence from our study and the studies of Mikheev *et al*. (2003) and Bandilla *et al*. (2005), that within natural water bodies the chances of an individual fish becoming infected with *A. foliaceus* are dependent mainly upon the risk of encountering this parasite rather than upon factors such as species preferences.

Q2) Are larval, juvenile and adult lice distributed similarly within the host community?

A2). No.

Large, slow moving, bottom feeding fish seem most prone to infection by adult lice whereas smaller slow moving fish that typically inhabit shallow littoral regions of a water body seem more prone to infection from larval lice (Chapters 3 and 4). The '*en-masse*' hatching of larval lice from egg strands deposited in these littoral regions can lead to large high infection intensities with some sticklebacks harbouring tens of larval lice and even as many as 143 on one specimen (Chapter 5).

Q3) Are larger fish more heavily infested with *A. foliaceus* than smaller fish?

A3) Partly.

Chapters 3, 4 and 5 provide an overview of the distribution of *A. foliaceus* on various fish species within a semi-natural water body. The results clearly indicate that within a mixed fish community certain fish individuals are more likely to become infested than others. It appears that size plays an important role in infection dynamics, as it does with many other ectoparasitic species (Cochrane, 1985; Poulin, 1993; Poulin, 1999; Poulin, 2000; Poulin *et al*., 1991; Rózsa, 1997; Tucker *et al*., 2000). The observed pattern is that larger fish are infested with higher numbers of lice. Preliminary investigations with *A. japonicus* infecting common

carp under controlled laboratory conditions suggest that this pattern of larger fish acquiring greater numbers of lice also holds true in aquaria. However, this does not necessarily mean that these larger fish are more heavily infested. At this stage we must consider the implications of the size of the parasite in relation to the host.

Lower numbers of lice do not necessarily infer less negative consequences for the host animal. Just one or two adult *Argulus* on a 3cm stickleback, for example, may have significantly higher, possibly even lethal, consequences. However, large carp can carry significantly higher numbers of lice without significant deterioration in condition (Chapter 4).

Survival

Q4) Does temperature affect the off-host survival time of *A. foliaceus* and *A. japonicus*?

A4) Yes.

During the search for a host, argulids survive as self-sufficient organisms either relying on stored energy in the form of yolk (newly hatched larvae) or their last meal (Chapter 2 and 6). The period of time these animals can survive without feeding on a host varies according to various published data (e.g. Shafir and Oldewage, 1992; Mikheev *et al*., 2003; Hakalhati *et al*., 2005). In Chapter 6 we investigated the effect of life history stage and temperature on the off-host survival and viability of *A. foliaceus* and *A. japonicus*. This chapter showed that temperature significantly affects the off-host survival of these two species. *A. japonicus* apparently had a higher tolerance to warmer temperatures than its native European counterpart *A. foliaceus* which appeared to survive better at slightly lower temperatures.

Q5) What are the maximum off-host survival times of these two species?

A5) *A. japonicus* = 13 days; *A. foliaceus* = 14 days.

The maximum survival times for these species were observed with adult lice. In Chapter 6 it has been shown that the three developmental stages (larval, juvenile and adult) all differ in terms of their off-host survival times with adults surviving longer than juveniles and juveniles surviving longer than larvae.

The difference in off-host survival times between species, life history stages and at different temperatures probably accounts for the varying reports within other published literature. It is hoped that the information presented in Chapter 6 will serve as more definitive reference guide for researchers working with these parasites in the future. Information of this kind may also prove useful in developing management strategies (e.g. fallowing of ponds and other fish rearing systems) to control these potentially devastating parasites. With the onset of

global warming *A. japonicus* may continue to extend its distribution northwards. More information regarding the biology and pathogenicity of this species compared to native *Argulus* spp. may prove vital in preventing serious economic damage to freshwater fish stocks in the future.

Attachment

Q6) Does temperature affect the attachment success of *A. japonicus*?

A6) Yes.

In Chapter 6 evidence was presented that at certain temperatures *A. japonicus* is more successful at locating and attaching to a host than at other temperatures. These "preferred" temperatures were similar to those reported as optimal temperatures for locomotion in *A. foliaceus* (Kollatsch, 1959). Such data are lacking for *A. japonicus*.

Q7) Does starvation affect the attachment success of *A. japonicus*?

A7) Yes

Data presented in Chapter 6 show that short periods of starvation do not significantly affect the attachment success of *A. japonicus*. For larval lice this was up to 2 days and for adult lice up to 3 days. After these short periods, however, there is a significant daily decrease in attachment success of these lice. After 5 days for larvae and 7 days for adults the parasites apparently do not have sufficient energy reserves for locomotion and consequently lose all ability to successfully locate a host fish and attach to it.

Feeding

Q8) What does *A. japonicus* actually eat?

A8) Whole blood (including intact erythrocytes)

Until recently it was difficult to find any clear statement regarding what these parasites actually eat (e.g. Kabata, 1970; Lamarre and Cochran, 1992; Gresty *et al*., 1993; Pasternak *et al*., 2000; van der Salm *et al*., 2000; Kearn, 2004). In Chapter 7 we discuss feeding in *A. japonicus* including recent reports by Tam and Avenant-Odewage (2006) regarding feeding in newly hatched argulids. In addition, the first evidence that an argulid (*A. japonicus*) does in fact consume whole blood, complete with whole erythrocytes, is presented. This has further implications for the development of anti-parasite treatments and the role of argulids as vectors for other pathogens that may be transmitted through fish blood (Ahne, 1985; Kearn, 2004 *and references therein*; Chapter 2 *and references therein*). In addition it is proposed that future

work should conduct similar investigations with other species of *Argulus* to evaluate how widespread hematophagy is within this parasite genus and outside of this genus in other branchiurans which lack the pre-oral stylet (e.g. *Dolops* spp.). If not, then it may provide further evidence that the pre-oral stylet is intimately involved in *Argulus* feeding activities perhaps by the introduction of a hemolytic substance and/or anti-coagulant from the glands described by Wilson (1903) that are located at the base of the stylet.

Host response

Q9) Does *A. japonicus* elicit an immune response in common carp?

A9) Yes.

Despite argulids being widely reported as causing significant damage to their hosts there is very little published material detailing the damage caused to the hosts by these animals feeding activities. There is also little known about immune responses of fish to these lice. Recent advances in the field of fish immunology have provided us with useful molecular tools to investigate immune responses to parasites in fish. The skin of fish typically provides the first line of defense against infectious agents. In spite of this fact there is very little information available relating to immune response mechanisms and associated gene transcription in the organ (Forlenza *et al*. 2008). In chapter 8 we provide an overview of immune responses elicited in the skin of juvenile common carp (*Cyprinus carpio*) by damage caused by the attachment and feeding activities of *A. japonicus*. Host fish for this study were infected with larval parasites and the results illustrate the timing of the immune response discussed in relation to the feeding habits of early life stages of this parasite species. An investigation into the effects of adult lice on the host would be most welcome but sadly was not possible within in the timeframe of this project mainly due to problems associated with the parasite culture. It is perceived that, because adults feed on whole blood and juveniles do not, host responses to adult lice feeding activities may be somewhat different to those occurring in response to feeding activities of early life stages of *A. japonicus*. The response of different host species to *Argulus* spp. has been reported to differ (Kaestner, 1967). Controlled infection studies comparing the responses of different host species to infection with *Argulus* are now required to evaluate such reports.

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Summaries

English Summary

Parasites of fish play a major role in the economic success of both fisheries and aquaculture operations. With the continuing expansion and diversification of these industries these parasites will continue impacting upon the economics of individual operations in a negative way. This highlights the importance of understanding the biology, ecology and host-parasite interaction of these animals so that we may ultimately develop management strategies that reduce the impact of these pathogens whilst inflicting minimal environmental and economic costs. In this thesis we have examined factors that influence the survival and distribution of an economically important ectoparasite genus found on freshwater fishes.

Argulus is the parasite that an angler or amateur naturalist is most likely to encounter in freshwater. This parasite has been reported in the past as exhibiting very low host specificity and will infect practically any species of fish that it encounters. In the past, and more recently, research has shown that *Argulus* is capable of decimating fish stocks and as such can be considered as a serious pest species. Despite the impact this parasite can have, there are still huge gaps in our understanding of its physiology, ecology and the effects it has upon the fish it infects. In **chapter 2** we reviewed the information currently available from published papers, reports and book chapters. This allowed us to identify key areas where details were lacking or conflicting.

In **chapters 3, 4** and **5** we explored the topic of host preference by examining the occurrence of native European argulid, *Argulus foliaceus*, on fish from a mixed species assemblage in a recreational angling lake. In **chapter 3** we found that *A. foliaceus* does not have an even distribution among host species within a single water body. Based on the parasite loads we concluded that slower moving, bottom feeding fish species (e.g. carp, *Cyprinus carpio* and bream, *Abramis brama*) were significantly more prone to infection than faster, surface feeding species (e.g. rudd, *Scardinius erythopthalmus*). In addition, we showed that three-spined sticklebacks (*Gasterosteus aculeatus*) were more prone to infection by larval *A. foliaceus* than any other species. We attributed the differential host-species utilization of *A. foliaceus* to the rate of host exposure to lice as determined by ecological and behavioural traits of both the hosts and parasites.

In **chapters 4** and **5** we examined whether host size influences the infection intensity of *A. foliaceus*. Larger hosts were significantly more likely to be infected by *A. foliaceus* and typically harboured significantly higher numbers of lice than their smaller counterparts. In **chapter 5** we also observed that below a certain size (approximately 3 cm) sticklebacks were

not found harbouring lice. The larger surface area of larger hosts is generally regarded as the determining factor behind why larger hosts are more heavily infected than smaller hosts. We hypothesised that behavioural differences between large and small fish might also account for some of the observed differences in infection levels, especially in the case of sticklebacks. We also pointed out that the consequences of infection for the host can be more severe for smaller hosts than larger hosts.

In **chapter 6** we observed how an abiotic factor, temperature, can significantly affect the off-host survival and viability of *Argulus* spp. We compared the off-host survival times at five different temperatures of larval, juvenile and adult lice from two *Argulus* species found in European freshwaters, the native *A. foliaceus* and the exotic invader *A. japonicus*. Temperature had a significant effect on the off-host survival times of both species. We also observed that the three developmental stages and the two species differed significantly in their off-host survival times at different temperatures. Starvation and temperature also had a significant effect on the attachment success of *A. japonicus*. We concluded that temperature is an important factor in determining the survival and viability of these two species and that global warming is likely to have important consequences for the future distribution of *A. japonicus*.

In **chapter 7** we explored a topic that has been debated frequently in the literature: what is the diet of *Argulus* spp.? We used a combination of morphological studies, live observations, histology and transmission electron microscopy to investigate the feeding mechanisms and diet of *A. japonicus*. We concluded that adult lice consume whole blood from their hosts and are probably predominantly hematophagous (blood feeders). The morphology of larval *A. japonicus*, and in particular the size of mouthparts, probably means these stages cannot ingest whole blood and as such feed on epithelial cells, and tissue fluids. We also showed that parasites can cause significant damage to the hosts epithelium, which results in red lesions occurring at sites where the parasites have been feeding.

In **chapter 8** we investigated the immune response of common carp (*Cyprinus carpio*) to infection from *A. japonicus*. Here we showed that the response of the fish is similar to the response caused by simple skin damage and therefore is probably not specific to the parasite. At sites where the parasite had been feeding inflammation was evident from a significant infiltration of leukocytes into the tissues surrounding the crater-like wound created by the feeding parasite.

This thesis has highlighted the need to continue investigations into this fascinating and economically important group of parasites. With the onset of global warming one is are likely

to see changes in the distribution of many parasite species including *Argulus* spp. As such it is important to rapidly gain an understanding of the ways in which these animals can affect their hosts. We must also continue to gather intelligence regarding these animals biology and how environmental factors such as temperature can affect this. This intelligence will undoubtedly prove vital in the future battle against these fish pests.

Samenvatting (in het Nederlands)

Visparasieten spelen een belangrijke rol bij het economische succes van zowel visserij als aquacultuur. In samenhang met de voortdurende uitbreiding en diversificatie van deze bedrijfstakken zijn het de parasieten die voortdurend een negatieve invloed uitoefenen op de opbrengst. Daarom is een beter begrip van de fysiologie, ecologie en gastheer-parasiet interacties van deze dieren van groot belang. Met behulp van deze toegenomen kennis zouden uiteindelijk strategieën ontwikkeld kunnen worden om de invloed van deze ziekteverwekkers te reduceren, tegen minimale milieu- en economische kosten. In dit proefschrift worden de factoren onderzocht die de overleving en verspreiding van een economisch belangrijk geslacht van ectoparasieten op vis (*Argulus*-soorten ofwel Karperluizen) bepalen.

Karperluizen zijn de parasieten die hengelaars of natuuronderzoekers vaak in zoet water tegenkomen. Deze parasieten zijn in het verleden als zeer weinig gastheerspecifiek beschouwd omdat ze praktisch elke soort vis die ze tegenkomen parasiteren. Uitvoerig onderzoek heeft aangetoond dat deze parasieten in staat zijn om de visstand te decimeren, en daarom kunnen ze worden beschouwd als zeer schadelijke soorten. Ondanks het feit dat de schadelijke invloed van deze parasieten goed bekend is, zijn er nog steeds grote leemten in onze kennis van hun fysiologie, ecologie, en van de effecten die deze dieren hebben op de vis die ze infecteren. In hoofdstuk 2 is de informatie samengevat die bij het begin van dit project beschikbaar was in de vorm van publicaties, rapporten en boekhoofdstukken. Hierdoor konden kennisgebieden waar details ontbraken of waar gegevens elkaar tegenspraken, worden opgespoord.

In de hoofdstukken 3, 4 en 5 wordt de gastheerpreferentie onderzocht van de inheemse Europese Karperluis *Argulus foliaceus* op vissen van een vissengemeenschap met meerdere soorten in een recreatieplas voor hengelaars. Eén van de conclusies in hoofdstuk 3 was dat *A. foliaceus* geen gelijke verdeling over de gastheervissen vertoont binnen eenzelfde waterplas. Op basis van de aantallen parasieten per vis is geconcludeerd dat langzamer zwemmende, benthivore vissoorten zoals karper (*Cyprinus carpio*) en brasem (*Abramis brama*) door meer parasieten geparasiteerd worden dan sneller zwemmende, zich meer aan het wateroppervlak voedende soorten zoals de rietvoorn (*Scardinius erythrophthalmus*). Bovendien bleek dat driedoornige stekelbaarzen (*Gasterosteus aculeatus*) meer geïnfecteerd worden door larvale *A. foliaceus* dan de andere onderzochte vissoorten. De verschillen in gastheergebruik door *A. foliaceus* kan worden geweten aan de frequentie van blootstelling aan de karperluizen,

hetgeen wordt bepaald door ecologische en gedragskenmerken van zowel gastheren als parasieten.

De hoofdstukken 4 en 5 behandelen de vraag of de grootte van de gastheren de infectie-intensiteit beïnvloedt. Grotere vissen waren significant meer geïnfecteerd door *A. foliaceus* en droegen doorgaans meer karperluizen bij zich dan hun kleinere soortgenoten. Onder een zekere grootte (ongeveer 3 cm) waren de driedoornige stekelbaarzen vrij van karperluizen. Hun grotere oppervlak wordt in het algemeen beschouwd als de reden dat grotere vissen meer geïnfecteerd worden dan kleine. Verschillen in gedrag tussen grote en kleine vissen zou ook de oorzaak kunnen zijn van de verschillen in infectieniveaus, vooral in het geval van driedoornige stekelbaarzen. Verder kunnen de consequenties van de infectie ernstiger zijn voor kleinere gastheren dan voor grotere.

In hoofdstuk 6 wordt behandeld hoe een abiotische factor, watertemperatuur, overleving en activiteit van *Argulus*-soorten zonder gastheer beïnvloedt. De overleving van karperluizen zonder gastheer is onderzocht voor larven, juvenielen en volwassen exemplaren van twee soorten karperluizen bij vijf verschillende temperaturen. De betreffen soorten zijn de inheemse *A. foliaceus* en de exotische invader *A. japonicus* die in Europa en elders zijn verspreidingsgebied uitbreidt. De watertemperatuur heeft een significant effect op de overlevingstijd van beide soorten. Bovendien is waargenomen dat bij verschillende temperaturen de drie ontwikkelingsstadia en de twee soorten zonder gastheer qua overlevingsduur significant verschilden. Uithongering en temperatuur hadden ook een duidelijk effect op het aanhechtingssucces van *A. japonicus*. De conclusie is dat de watertemperatuur de overleving en levensvatbaarheid van deze twee soorten bepaalt en dat daarom opwarming van de aarde waarschijnlijk belangrijk is voor de toekomstige verspreiding van, vooral, *A. japonicus*.

In hoofdstuk 7 wordt een onderwerp geëxploreerd dat vaak het onderwerp van debat in de literatuur is, namelijk het dieet van karperluizen. Voor deze studie is een combinatie gebruikt van observaties aan levende dieren, histologie en transmissie electronenmicroscopie, teneinde de voedingsmechanismen en het dieet van *A. japonicus* vast te stellen. Volwassen karperluizen van deze soort consumeren bloed inclusief de rode bloedcellen en zijn dus vooral bloedzuigers. De morfologie van de larven van deze soorten, in het bijzonder de grootte van de monddelen, maakt het hen onmogelijk om het bloed te benutten. Ze voeden zich waarschijnlijk met epitheelcellen en weefselvloeistof. Ook is aangetoond dat de parasieten veel schade kunnen aanrichten aan het huidepitheel, wat resulteert in rode laesies op plekken waar de parasieten zich gevoed hebben.

In hoofdstuk 8 is de immuunrespons van de gewone karper (*Cyprinus carpio*) op infectie door *A. japonicus* bestudeerd. Deze respons lijkt sterk op de respons die veroorzaakt wordt door een simpele huidbeschadiging, en is dus niet specifiek voor de parasiet. Op plekken waar de parasiet zich had gevoed was inflammatie duidelijk zichtbaar als een infiltratie van leucocyten in een kratervormige wond die veroorzaakt is door de zich voedende parasiet.

Dit proefschrift maakt duidelijk dat onderzoek naar deze fascinerende en economisch belangrijke groep van parasieten zou moeten worden voortgezet. De opwarming van de aarde zal veranderingen veroorzaken in de verspreiding van vele soorten inclusief parasieten waaronder *Argulus*-soorten. Daarom is het belangrijk om meer kennis te verzamelen over hoe deze dieren hun gastheren kunnen aantasten. We moeten ook meer kennis vergaren omtrent de biologie van deze dieren en hoe omgevingsfactoren zoals temperatuur op hen inwerken. Zulke kennis zal ongetwijfeld van groot belang zijn bij de toekomstige bestrijding van visplagen.

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one of the most memorable experiences of my life and is something I would not have enjoyed without you pushing me to do it. There will be a blanket and cookies for you wherever I live in the future. Bevelander, it seems very fitting that you're with a girl that can cook such great food having seen how much you enjoy eating! As with Mari, I considered you a part of my family rather than a friend. You fit the role of "big brother" perfectly and I enjoyed having someone around that loved a good fight as much as I did, whether it was on the screen or on the mats. Thanks to my fellow Zanshin members, especially Gideon, Christian, Bas and Ralph for the extra lunchtime training sessions, you all taught me so much.

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Curriculum vitae

Peter Walker was born on the 2nd of January 1979 in Blackpool, the UK and grew up in the small fishing town of Fleetwood. His parents, Kathy and Stewart Walker recognized from an early age that Peter was likely to pursue a career working with animals having seen the constant stream of invasive species entering their own ecosystem with some anthropogenic assistance from Peter. Bugs (invertebrates) seemed to be the creatures that fascinated Peter the most, that was until he developed a passion for angling. With a passion for invertebrates and fish, a career in fish parasitology was not surprising because this research field offered a perfect way to combine both groups of animals for research purposes. *Argulus* featured in Peter's academic career relatively early on. For his A-level (College) biology project Peter studied host choice by this animal and whilst the experiments conducted were of a relatively novice standard the ideas and results were remarkably interesting. It was during these investigations at just 17years old that Peter recognized a distinct gap in the knowledge surrounding these animals. This planted the seed that there was an opportunity to be grasped here. Upon the completion of his A-levels Peter embarked on his university career by studying Marine and Freshwater Biology at The University of Wales Aberystwyth, UK. Upon the completion of his bachelors degree Peter was awarded a Walter-Idris summer research internship at Aberystwyth working under the supervision of Dr Iain Barber. Here he undertook investigations examining the way in which parasitic tapeworms affect the behaviour of the hosts. This resurrected his passion for parasites and when he began the Masters course in Applied Fish Biology at Plymouth University, UK, there was no doubt surrounding his choice of research project. It was here that the he became reacquainted with *Argulus* and contacts formed during this research project resulted in him being offered the position of Junior Researcher investigating *Argulus* at the Radboud University Nijmegen, The Netherlands. Peter is currently working as a Research Officer at The Bangor University in Wales (UK) and is an Advisory Editor of the scientific journal, Crustaceana. From August 2008, he will be employed as an Aquatic Ecologist by APEM, an aquatic consultancy organisation based in Oxford.

Publications

Peer-reviewed papers

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*Forlenza, M., ***Walker, P. D.**, de Vries, B. J., Wendelaar Bonga, S. E. & Wiegertjes, G. F. Transcriptional analysis of the common carp (*Cyprinus carpio* L.) immune response to the fish louse *Argulus japonicus* Thiele (Crustacea: Branchiura). *Fish and Shellfish Immunology* (in press)

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Book Chapter

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